IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Ryozo NAGAI et al.

Group Art Unit : 1611

Appl. No. : 10/598,275

Examiner : Kyle Purdy

I. A. Filed : February 24, 2005

Confirmation No. : 2170

For : MEDICINE CAPABLE OF INHIBITING ACTIVATION OF TRANSCRIPTION FACTOR KLF5

APPEAL BRIEF UNDER 37 C.F.R. § 41.37

Commissioner for Patents
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401 Dulany Street
Alexandria, VA 22314

Sir :

This appeal is under 35 U.S.C. 134 from the decision of the Examiner finally rejecting claims 24, 28, 29, 32, 33 and 37 as forth in the Final Office Action dated September 2, 2010 as modified by the Advisory Action dated February 2, 2011 and the Examiner Interview Summary form dated March 30, 2011 making of record a telephone interview of March 23, 2011.

A Notice of Appeal to the September 2, 2010 Final Office Action has been filed on February 25, 2011 so that the time for filing an Appeal Brief extends until April 25, 2011.

Appellant notes that this Appeal Brief is being filed by April 25, 2011 so that an extension of time and the fee associated therewith should not be necessary for maintaining the pendency of the application. However, if for any reason any extension of time and/or any fee is necessary to maintain the pendency of the application, including any extension of time and/or any appeal fee, this is an express request for any required extension of time and authorization to charge any necessary fee to Deposit Account No. 19-0089.

The requisite fee under 37 C.F.R. 41.20(b)(2) in the amount of $540.00 for the filing of the Appeal Brief is being paid herewith.

As noted above, if for any reason any extension of time and/or any fee is required to maintain the pendency of the application, including any extension of time and/or appeal fee, authorization is hereby provided to charge any required fee, including any fee for the Appeal Brief and any necessary extension of time fee to Deposit Account No. 19-0089.

(I) REAL PARTY IN INTEREST

The real party in interest is The University of Tokyo and Kowa Pharmaceutical Co., Ltd. by at assignment by an assignment from the inventors recorded March 28, 2007, at Reel 019117, Frame and an assignment from Kowa Pharmaceutical Co., Ltd. to Kowa Company, Ltd. recorded at Reel 02161, Frame 0973.

(II) RELATED APPEALS AND INTERFERENCES

None

There are no pending related appeals and/or interferences.

(III) STATUS OF CLAIMS

The status of the claims is as follows:

Claims 24, 28, 29, 32, 33 and 37 are pending in this application and are under appeal.

Claims 1-23, 25-27, 30, 31 and 34-36 and 38-48 are canceled.

Of the pending claims, claims 24, 28, 29, 32, 33 and 37 have been finally rejected in the Final Office Action dated September 2, 2010 as modified by the Advisory Action dated February 2, 2011 and the Examiner Interview Summary form dated March 30, 2011 making of record a telephone interview of March 23, 2011. In this regard, the Examiner Interview Summary states that claims 24, 28, 29, 32, 33 and 37 were discussed, but includes a typographical error in listing claim 34 instead of claim 33 in the Substance of Interview section of the form. A Statement of Interview was filed April 18, 2011 by Appellant.

The finally rejected claims are included in the listing of Claims Under Appeal.

(IV) STATUS OF AMENDMENTS

The appeal is based upon finally rejected claims.

An amendment was filed January 18, 2011 wherein claims 24, 28, 29, 32, 33 and 37 were amended, and claims 34-36 and 38-48 were canceled.

An Advisory Action dated February 2, 2011 entered Appellant's amendment filed January 18, 2011 and was clarified in the Examiner Interview Summary dated March 30, 2011 making of record a March 23, 2011 telephone interview so that the finally rejected claims are claims 24, 28, 29, 32, 33 and 37 as amended in the response filed January 18, 2011. In this regard, the Examiner Interview Summary states that claims 24, 28, 29, 32, 33 and 37 were discussed, but includes a typographical error in listing claim 34 instead of claim 33 in the Substance of Interview section of the form. A Statement of Interview was filed April 18, 2011 by Appellant.

(V) SUMMARY OF THE CLAIMED SUBJECT MATTER

The following description is made with respect to the independent claims and includes references to particular parts of the specification. As such, the following is merely exemplary and is not a surrender of other aspects of the present invention that are also enabled by the present specification and that are directed to equivalent methods within the scope of the claims.

Independent Claim 24

Independent claim 24 recites a method of treatment for arteriosclerosis (e.g., page 4, paragraph [0010], including lines 21-23 thereof), comprising administering to a mammal in need of treatment (e.g., page 4, paragraph [0010], including lines 15-23; and pages 6 and 7, paragraph [[0016], including page 6, line 6, line 27 to page 7, line 9) a medicament comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient (e.g., page 4, paragraph [0009], including lines 3-13), such that activation of a transcription factor KLF5 is inhibited and/or such that vascular remodeling is inhibited (e.g., page 4, paragraph [0010], including lines 15-23; and pages 6 and 7, paragraph [[0016], including page 6, line 6, line 27 to page 7, line 9).

Independent Claim 33

Independent claim 33 recites a method of inhibiting activation of a transcription factor KLF5 (e.g., page 3, paragraph [0007], including lines 20-28 thereof; and page 4, paragraph [0010], including lines 16-19 thereof), comprising contacting one or more cells which express KLF5 with a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient (e.g., page 4, paragraph [0009], including lines

3-13) such that activation of KLF5 is inhibited (e.g., Appellant's Examples on page 7 line 23 to page 11, line 27).

Independent Claim 37

Independent claim 37 recites a method of inhibiting vascular remodeling (e.g., page 4, paragraph [0010], including lines 19-21 thereof), comprising administering to a mammal a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient (e.g., page 4, paragraph [0009], including lines 3-13) such that vascular remodeling is inhibited.

(VI) GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claims 24, 28, 29, 32, 33 and 37 are rejected under 35 U.S.C. 103(a) as being unpatentable over Marx et al., *Circ. Res.* **90**:703-710, 2002 (hereinafter referred to as "Marx") in view of Shidoji et al., WO 01/80854, as evidenced by the English equivalent US 2005/0250671 A1 (and hereinafter collectively referred to as "Shidoji" and with reference being made to the disclosure as set forth in US 2005/0250671 A1).

(VII) ARGUMENT

(I) Traversal of rejection of claim 24 under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji.

(a) Claim 24 is not properly rejected under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji.

(A) Arguments for Independent Claim 24

The rejection of independent claim 24 under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji is in error, the decision of the Examiner to reject this claim should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 24 is directed to a method of treatment for arteriosclerosis, comprising administering to a mammal in need of treatment a medicament comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid (hereinafter also referred to as "NIK-333") as an active ingredient such that activation of a transcription factor KLF5 is inhibited and/or such that vascular remodeling is inhibited.

Appellant submits that there is no reason to arrive at Appellant's claimed subject matter based upon any combination of Marx and Shidoji. Marx in view of Shidoji fails to teach or suggest a method wherein the activation of a transcription factor KLF5 is inhibited and/or wherein vascular remodeling is inhibited let alone a method of treatment for arteriosclerosis, comprising administering to a mammal in need of treatment a medicament comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that activation of a transcription factor KLF5 is inhibited and/or such that vascular remodeling is inhibited. There is no direction in either of Marx or Shidoji and/or any combination of Marx and Shidoji to arrive at a method of treatment for arteriosclerosis in a

mammal in need thereof using NIK-333, to arrive at a method of administering NIK-333 to a mammal in need of treatment let alone administering NIK-333 to a mammal in need of treatment such that such that activation of a transcription factor KLF5 is inhibited and/or such that vascular remodeling is inhibited.

Still further, while even the combined disclosures of Marx and Shidoji do not teach or suggest Appellant's claimed subject matter, there is no reason why one having ordinary skill in the art would have sought to combine the disclosures of Marx and Shidoji. One having ordinary skill in the art would not have sought to combine the teachings of Marx with those of Shidoji at least because Shidoji and Marx are directed to disparate subject matter. Marx is a research report which discloses the results of experiments performed to determine the effect of PPAR activators on CD4+ T cells. For example, the abstract of Marx, beginning in line 5, discloses:

> The present study investigated whether activators of peroxisome proliferator-activated receptor (PPAR)α and PPARγ, with their known antiinflammatory effects, might regulate the expression of proinflammatory cytokines in human CD4-positive T cells.

In contrast, Shidoji is directed to activators of peroxisome proliferator-activated receptors comprising a polyprenyl compound, preferably (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, as an active ingredient, and medicaments for preventive and/or therapeutic treatment of hyperlipidemia, non-insulin dependent diabetes mellitus or the like comprising a polyprenyl compound as an active ingredient. Therefore, Shidoji discloses the use of PPAR activators in the treatment of hyperlipidemia and non-insulin dependent diabetes. Thus, not only would one having ordinary skill in the art not have combined the cited art in the manner contended in the rejection, it would not have been obvious to treat arteriosclerosis with NIK-333 absent knowledge that such a compound inhibits KLF5 and/or inhibits vascular remodeling.

In the rejection, the Examiner asserts that inhibition of vascular remodeling would be obvious because "...Marx teaches that PPAR activators (i.e. 3,7,11,15-tetramethyl-2,4,6,10,14 hexadecapentanoic acid) are useful for reducing inflammation in transplant associated arteriosclerosis." (See page 4, Section 10 of the Final Office Action). However, even if Marx discloses that PPAR activators are useful for reducing inflammation in transplant-associated arteriosclerosis, this does not mean that Marx discloses inhibition of vascular remodeling, either explicitly or inherently. Indeed, the Examiner appears to concede at page 6, section 16 of the Office Action that any reduction in the expression of proinflammatory cytokines from the activation of PPAR yields only "potential" therapeutic benefits in pathological processes such as atherosclerosis and transplantation-associated arteriosclerosis.

The Examiner appears to be asserting that one of ordinary skill in the art could envisage use of NIK-333 in the method of Marx with a reasonable expectation of success (see Office Action at page 4, section 11, and pages 6-7, section 19). However, use of NIK-333 in the method of Marx would result in pre-treatment of human CD4-positive T cells in culture with NIK-333, not administration of NIK-333 to a mammal in need of treatment for arteriosclerosis as claimed. Thus, Appellant's claimed subject matter cannot be achieved by a simple substitution of NIK-333 in the method of Marx as the Examiner appears to assert.

During an October 27, 2010 telephone interview, the Examiner contended that Marx discloses, at page 704, left column, first full paragraph, that given the role of T-lymphocyte inflammatory cytokine production in atherosclerosis and evidence of PPARs as anti-inflammatory mediators, it is hypothesized that human T lymphocytes express PPARα and PPARγ and that stimulation of these cells by PPAR activators in clinical use would limit inflammatory cytokine expression. Moreover, the Examiner contended that Shidoji discloses

that acyclic polyprenyl compounds are activators of PPAR. The Examiner contended that in view of such disclosure there is a reasonable expectation of success of using the acyclic polyprenyl compounds of Shidoji with the disclosure of Marx to treat atherosclerosis and Appellant's recited subject matter would be at hand.

With regard to the above, Appellant submits that there is motivation for one having ordinary skill in the art to combine the disparate disclosures of Marx and Shidoji for at least the reasons set forth above. However, even if for the sake of argument a *prima facie* case of obviousness has been established, Appellant's claimed subject matter provides unexpected advantages. As shown below, (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid (NIK-333), as recited in Appellant's claims provides advantageous effects as shown with respect to ATRA (all-trans retinoic acid) in view of the experimental results in Example 5 of Appellant's specification for KLF5 inhibition and Example 3-2 for vascular remodeling.

As can be understood from the experimental results of Example 5, at page 11 of Appellant's specification and as illustrated in Fig. 5, NIK-333 provides more potent inhibitory action than ATRA (which was used as a comparative retinoid) against the proliferation of the tested 3T3-KLF5 cells in which KLF5 were stably expressed. In this regard, KLF5 has the action of stimulating proliferation of cells (see, for example, Miyamoto et al., Molecular and Cellular Biology, Vol. 23, No. 23, Dec. 2003, pp. 8528-8541, such as in the abstract thereof, at lines 4 and 5). In contrast, prior to Appellant's invention, it was known in the field of art that the pharmacological action of ATRA via the retinoid receptor is more potent than NIK-333 (see, Tsurumi et al., International Journal of Hematology, 59, pp. 9-15, 1993 wherein E5166 corresponds to NIK-333). Therefore, the experimental results of Example 5 in Appellant's

specification as originally filed establish unexpected results of Appellant's recited (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid. Accordingly, one having ordinary skill in the art would not have expected the results as illustrated in Appellant's originally filed application.

Further, the action of NIK-333 against vascular remodeling was revealed to be more potent than ATRA in Example 3-2 of Appellant's specification by using the mouse cuff-induced injury model, as see pages 9 and 10, and Table 2 of Appellant's specification. Accordingly, one of ordinary skill in the art would not have been able to expect the superior action of NIK-333 than ATRA.

Accordingly, at least for the reasons set forth above, the rejection of claim 24 is without appropriate basis and should be reversed.

(II) Traversal of rejection of claim 33 under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji.

(a) Claim 33 is not properly rejected under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji.

(A) Arguments for Independent Claim 33

The rejection of independent claim 33 under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji is in error, the decision of the Examiner to reject this claim should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 33 is directed to is directed to a method of inhibiting activation of a transcription factor KLF5, comprising contacting one or more cells which express

KLF5 with a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that activation of KLF5 is inhibited.

Appellant submits that there is no reason to arrive at Appellant's claimed subject matter based upon any combination of Marx and Shidoji. Marx in view of Shidoji fails to teach or suggest a method wherein one or more cells which express KLF5 are contacted with a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that activation of KLF5 is inhibited.

There is no direction in either of Marx or Shidoji and/or any combination of Marx and Shidoji to arrive at a method of inhibiting activation of a transcription factor KLF5 using NIK-333.

Still further, while even the combined disclosures of Marx and Shidoji do not teach or suggest Appellant's claimed subject matter, there is no reason why one having ordinary skill in the art would have sought to combine the disclosures of Marx and Shidoji. One having ordinary skill in the art would not have sought to combine the teachings of Marx with those of Shidoji at least because Shidoji and Marx are directed to disparate subject matter. Marx is a research report which discloses the results of experiments performed to determine the effect of PPAR activators on CD4+ T cells. For example, the abstract of Marx, beginning in line 5, discloses:

> The present study investigated whether activators of peroxisome proliferator-activated receptor (PPAR)α and PPARγ, with their known antiinflammatory effects, might regulate the expression of proinflammatory cytokines in human CD4-positive T cells.

In contrast, Shidoji is directed to activators of peroxisome proliferator-activated receptors comprising a polyprenyl compound, preferably (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, as an active ingredient, and medicaments for preventive and/or therapeutic treatment of hyperlipidemia, non-insulin dependent diabetes mellitus or the

like comprising a polyprenyl compound as an active ingredient. Therefore, Shidoji discloses the use of PPAR activators in the treatment of hyperlipidemia and non-insulin dependent diabetes. Thus, not only would one having ordinary skill in the art not have combined the cited art in the manner contended in the rejection, it would not have been obvious to treat arteriosclerosis with NIK-333 absent knowledge that such a compound inhibits KLF5 and/or inhibits vascular remodeling.

During an October 27, 2010 telephone interview, the Examiner contended that Marx discloses, at page 704, left column, first full paragraph, that given the role of T-lymphocyte inflammatory cytokine production in atherosclerosis and evidence of PPARs as anti-inflammatory mediators, it is hypothesized that human T lymphocytes express PPARα and PPARγ and that stimulation of these cells by PPAR activators in clinical use would limit inflammatory cytokine expression. Moreover, the Examiner contended that Shidoji discloses that acyclic polyprenyl compounds are activators of PPAR. The Examiner contended that in view of such disclosure there is a reasonable expectation of success of using the acyclic polyprenyl compounds of Shidoji with the disclosure of Marx to treat atherosclerosis and Appellant's recited subject matter would be at hand. However, this does not address Appellant's recited method in claim 33 which is directed a method of inhibiting activation of a transcription factor KLF5 comprising contacting one or more cells which express KLF5 with a composition comprising NIK-333 as an active ingredient such that activation of KLF5 is inhibited..

Moreover, Appellant submits that there is no motivation for one having ordinary skill in the art to combine the disparate disclosures of Marx and Shidoji for at least the reasons set forth above. However, even if for the sake of argument a *prima facie* case of obviousness has been established, Appellant's claimed subject matter provides unexpected advantages. As shown

below, (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid (NIK-333), as recited in Appellant's claims provides advantageous effects as shown with respect to ATRA (all-trans retinoic acid) in view of the experimental results in Example 5 of Appellant's specification for KLF5 inhibition.

As can be understood from the experimental results of Example 5, at page 11 of Appellant's specification and as illustrated in Fig. 5, NIK-333 provides more potent inhibitory action than ATRA (which was used as a comparative retinoid) against the proliferation of the tested 3T3-KLF5 cells in which KLF5 were stably expressed. In this regard, KLF5 has the action of stimulating proliferation of cells (see, for example, Miyamoto et al., Molecular and Cellular Biology, Vol. 23, No. 23, Dec. 2003, pp. 8528-8541, such as in the abstract thereof, at lines 4 and 5). In contrast, prior to Appellant's invention, it was known in the field of art that the pharmacological action of ATRA via the retinoid receptor is more potent than NIK-333 (see, Tsurumi et al., International Journal of Hematology, 59, pp. 9-15, 1993 wherein E5166 corresponds to NIK-333). Therefore, the experimental results of Example 5 in Appellant's specification as originally filed establish unexpected results of Appellant's recited (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid. Accordingly, one having ordinary skill in the art would not have expected the results as illustrated in Appellant's originally filed application.

Accordingly, at least for the reasons set forth above, the rejection of claim 33 is without appropriate basis and should be reversed.

(III) Traversal of rejection of independent claim 37 and dependent claims 28, 29 and 32 under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji.

(a) Claims 28, 29, 32 and 37 are not properly rejected under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji.

(A) Arguments for Independent Claim 37 and Dependent Claims 28, 29 and 32

The rejection of independent claim 37 and dependent claims 28, 29 and 32 under 35 U.S.C. 103(a) as being unpatentable over Marx in view of Shidoji is in error, the decision of the Examiner to reject this claim should be reversed, and the application should be remanded to the Examiner.

Appellant's independent claim 37 is directed to a method of inhibiting vascular remodeling, comprising administering to a mammal a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that vascular remodeling is inhibited.

Appellant submits that there is no reason to arrive at Appellant's claimed subject matter based upon any combination of Marx and Shidoji. Marx in view of Shidoji fails to teach or suggest a method of inhibiting vascular remodeling, comprising administering to a mammal a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that vascular remodeling is inhibited. There is no direction in either of Marx or Shidoji and/or any combination of Marx and Shidoji to arrive at a method of inhibiting vascular remodeling using NIK-333 in a mammal such that vascular remodeling is inhibited.

Still further, while even the combined disclosures of Marx and Shidoji do not teach or suggest Appellant's claimed subject matter, there is no reason why one having ordinary skill in

the art would have sought to combine the disclosures of Marx and Shidoji. One having ordinary skill in the art would not have sought to combine the teachings of Marx with those of Shidoji at least because Shidoji and Marx are directed to disparate subject matter. Marx is a research report which discloses the results of experiments performed to determine the effect of PPAR activators on CD4+ T cells. For example, the abstract of Marx, beginning in line 5, discloses:

> The present study investigated whether activators of peroxisome proliferator-activated receptor (PPAR)α and PPARγ, with their known antiinflammatory effects, might regulate the expression of proinflammatory cytokines in human CD4-positive T cells.

In contrast, Shidoji is directed to activators of peroxisome proliferator-activated receptors comprising a polyprenyl compound, preferably (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid, as an active ingredient, and medicaments for preventive and/or therapeutic treatment of hyperlipidemia, non-insulin dependent diabetes mellitus or the like comprising a polyprenyl compound as an active ingredient. Therefore, Shidoji discloses the use of PPAR activators in the treatment of hyperlipidemia and non-insulin dependent diabetes. Thus, not only would one having ordinary skill in the art not have combined the cited art in the manner contended in the rejection, it would not have been obvious to inhibit vascular remodeling with NIK-333 in a mammal.

In the rejection, the Examiner asserts that inhibition of vascular remodeling would be obvious because "...Marx teaches that PPAR activators (i.e. 3,7,11,15-tetramethyl-2,4,6,10,14 hexadecapentanoic acid) are useful for reducing inflammation in transplant associated arteriosclerosis." (See page 4, Section 10 of the Final Office Action). However, even if Marx discloses that PPAR activators are useful for reducing inflammation in transplant-associated arteriosclerosis, this does not mean that Marx discloses inhibition of vascular remodeling, either explicitly or inherently. Indeed, the Examiner appears to concede at page 6, section 16 of the

Office Action that any reduction in the expression of proinflammatory cytokines from the activation of PPAR yields only "potential" therapeutic benefits in pathological processes such as atherosclerosis and transplantation-associated arteriosclerosis.

The Examiner appears to be asserting that one of ordinary skill in the art could envisage use of NIK-333 in the method of Marx with a reasonable expectation of success (see Office Action at page 4, section 11, and pages 6-7, section 19). However, use of NIK-333 in the method of Marx would result in pre-treatment of human CD4-positive T cells in culture with NIK-333, not administration of NIK-333 to a mammal in need of treatment for arteriosclerosis as claimed. Thus, Appellant's claimed subject matter cannot be achieved by a simple substitution of NIK-333 in the method of Marx as the Examiner appears to assert.

During an October 27, 2010 telephone interview, the Examiner contended that Marx discloses, at page 704, left column, first full paragraph, that given the role of T-lymphocyte inflammatory cytokine production in atherosclerosis and evidence of PPARs as anti-inflammatory mediators, it is hypothesized that human T lymphocytes express PPARα and PPARγ and that stimulation of these cells by PPAR activators in clinical use would limit inflammatory cytokine expression. Moreover, the Examiner contended that Shidoji discloses that acyclic polyprenyl compounds are activators of PPAR. The Examiner contended that in view of such disclosure there is a reasonable expectation of success of using the acyclic polyprenyl compounds of Shidoji with the disclosure of Marx to treat atherosclerosis and Appellant's recited subject matter would be at hand.

With regard to the above, Appellant submits that there is no motivation for one having ordinary skill in the art to combine the disparate disclosures of Marx and Shidoji for at least the reasons set forth above. However, even if for the sake of argument a *prima facie* case of

obviousness has been established, Appellant's claimed method of inhibiting vascular remodeling would not be at hand because there is no teach or suggestion of a method of inhibiting vascular remodeling using NIK-333.

Still further, Appellant's claimed subject matter provides unexpected advantages. As shown below, (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid (NIK-333), as recited in Appellant's claims provides advantageous effects as shown with respect to ATRA (all-trans retinoic acid) in view of the experimental results in Example 3-2 for vascular remodeling.

As can be understood from the experimental results of Example 3-2, the action of NIK-333 against vascular remodeling was revealed to be more potent than ATRA (which was used as a comparative retinoid). In this regard, the action of NIK-333 against vascular remodeling was revealed to be more potent than ATRA in Example 3-2 of Appellant's specification by using the mouse cuff-induced injury model, as see pages 9 and 10, and Table 2 of Appellant's specification. Accordingly, one of ordinary skill in the art would not have been able to expect the superior action of NIK-333 than ATRA.

Accordingly, one having ordinary skill in the art would not have expected the results as illustrated in Appellant's originally filed application.

Therefore, at least for the reasons set forth above, the rejection of claim 37 is without appropriate basis and should be reversed.

Still further, dependent claims 28, 29 and 32 are patentable over any combination of Marx and Shidoji at least for the reasons presented with respect to claim 37.

Accordingly, the rejection of claims 28, 29 and 32 is without appropriate basis and should be reversed.

CONCLUSION

For the reasons set forth above, it is respectfully submitted that the Examiner has failed to establish that a *prima facie* case of obviousness is present, which is a prerequisite for maintaining a rejection under 35 U.S.C. 103(a). Moreover, Appellant's showing of unexpected results should overcome any *prima facie* case of obviousness even if present. The Board is, therefore, respectfully requested to reverse the Final Rejection, and to allow the application to issue in its present form.

Respectfully submitted,
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Attachments: (VIII) Claims Appendix
(IX) Evidence Appendix
(X) Related Proceedings Appendix

(VIII) CLAIMS APPENDIX

CLAIMS ON APPEAL

24. A method of treatment for arteriosclerosis, comprising administering to a mammal in need of treatment a medicament comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that activation of a transcription factor KLF5 is inhibited and/or such that vascular remodeling is inhibited.

28. The method according to claim 37, wherein the composition is in the form of a pharmaceutical composition containing a pharmaceutically acceptable additive together with the (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient.

29. The method according to claim 37, wherein the composition is in the form of a pharmaceutical composition for oral administration.

32. The method according to claim 37, wherein the mammal is a human.

33. A method of inhibiting activation of a transcription factor KLF5, comprising:

contacting one or more cells which express KLF5 with a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that activation of KLF5 is inhibited.

37. A method of inhibiting vascular remodeling, comprising:

administering to a mammal a composition comprising (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as an active ingredient such that vascular remodeling is inhibited.

(IX) Evidence Appendix

Copies of evidence entered by the Examiner and relied upon by Appellant in the appeal along with statements setting from where in the record that evidence was entered in the record by the Examiner.

Marx et al., *Circ. Res.* **90**:703-710, 2002 - entered in the record in Form PTO-892 attached to the Office Action dated March 30, 2010.

WO 01/80854 A1 to Shidoji et al. as evidenced by the English equivalent US 2005/0250671 A1 – entered in the record in the Form PTO-892 attached to the Office Action date March 30, 2010.

Miyamoto et al., Molecular and Cellular Biology, Vol. 23, No. 23, Dec. 2003, pp. 8528-8541 – entered in the record in Appellant's Amendment Under 37 C.F.R. 1.116, filed January 18, 2011 with the response being entered into the record in the Advisory Action dated February 2, 2011.

Tsurumi et al., International Journal of Hematology, 59, pp. 9-15, 1993 – entered in the record in Appellant's Amendment Under 37 C.F.R. 1.116, filed January 18, 2011 with the response being entered into the record in the Advisory Action dated February 2, 2011.

(X) Related Proceedings Appendix

None

Circulation Research

American Heart Association.
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PPAR Activators as Antiinflammatory Mediators in Human T Lymphocytes: Implications for Atherosclerosis and Transplantation-Associated Arteriosclerosis

Nikolaus Marx, Bettina Kehrle, Klaus Kohlhammer, Miriam Grüb, Wolfgang Koenig, Vinzenz Hombach, Peter Libby and Jorge Plutzky

Circ. Res. 2002;90;703-710; originally published online Feb 28, 2002;
DOI: 10.1161/01.RES.0000014225.20727.8F
Circulation Research is published by the American Heart Association. 7272 Greenville Avenue, Dallas, TX 72514
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PPAR Activators as Antiinflammatory Mediators in Human T Lymphocytes

Implications for Atherosclerosis and Transplantation-Associated Arteriosclerosis

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Abstract—Activation of T lymphocytes and their ensuing elaboration of proinflammatory cytokines, such as interferon (IFN)-γ, represent a critical step in atherogenesis and arteriosclerosis. IFNγ pathways also appear integral to the development of transplantation-associated arteriosclerosis (Tx-AA), limiting long-term cardiac allograft survival. Although disruption of these IFNγ signaling pathways limits atherosclerosis and Tx-AA in animals, little is known about inhibitory regulation of proinflammatory cytokine production in humans. The present study investigated whether activators of peroxisome proliferator–activated receptor (PPAR)α and PPARγ, with their known antiinflammatory effects, might regulate the expression of proinflammatory cytokines in human CD4-positive T cells. Isolated human CD4-positive T cells express PPARα and PPARγ mRNA and protein. Activation of CD4-positive T cells by anti-CD3 monoclonal antibodies significantly increased IFNγ protein secretion from 0 to 504±168 pg/mL, as determined by ELISA. Pretreatment of cells with well-established PPARα (WY14643 or fenofibrate) or PPARγ (BRL 49653/rosiglitazone or pioglitazone) activators reduced anti-CD3–induced IFNγ secretion in a concentration-dependent manner. PPAR activators also inhibited TNFα and interleukin-2 protein expression. In addition, PPAR activators markedly reduced cytokine mRNA expression in these cells. Such antiinflammatory actions were also evident in cell-cell interactions with medium conditioned by PPAR activator–treated T cells attenuating human monocyte CD64 expression and human endothelial cell major histocompatibility complex class II induction. Thus, activation of PPARα and PPARγ in human CD4-positive T cells limits the expression of proinflammatory cytokines, such as IFNγ, yielding potential therapeutic benefits in pathological processes, such as atherosclerosis and Tx-AA. (*Circ Res.* **2002;90:703-710.**)

Key Words: atherosclerosis ■ fibrates ■ thiazolidinediones ■ peroxisome proliferator–activated receptors ■ T cells

The activation of T lymphocytes contributes importantly to atherogenesis.[1,2] In human atheroma, CD4-positive cells, the major T-cell population, appear to promote atherosclerosis through their elaboration of proinflammatory cytokines, such as interferon (IFN) γ, tumor necrosis factors (TNFs), and interleukin (IL)-2.[1,3,4] These cytokines contribute to plaque development through their activation of endothelial cells (ECs) and modulation of macrophage and vascular smooth muscle cell responses.[5,6] Indeed, patients with atherosclerosis and acute coronary syndromes exhibit T-cell activation and increased IFNγ serum levels.[7,8] In apoE-deficient mice, interruption of the IFNγ signaling pathway reduces the extent of atherosclerotic lesions.[9] Similar proinflammatory effects of T-lymphocyte–derived cytokines participate in transplantation-associated arteriosclerosis (Tx-AA), a disease accounting for most cardiac transplantation failures.[10] In various animal models of transplantation, decreased or absent IFNγ production limited subsequent allograft vasculopathy.[11] Despite this large body of data implicating IFNγ in atherosclerosis and Tx-AA, pathways that might limit inflammatory cytokine production by human lymphocytes remain largely unexplored in the context of vascular disease.

Recent work from several groups implicates the nuclear receptors peroxisome proliferator–activated receptor (PPAR)α and PPARγ as antiinflammatory mediators in atheroma-associated cells.[12–17] PPARs, like other nuclear receptors, regulate gene expression through their actions as transcription factors in response to specific ligands.[18] PPARα activators include lipid-lowering fibric acid derivatives, such as fenofibrate or WY14643, and certain polyunsaturated fatty acids.[19] PPARγ ligands include the thiazolidinedione (TZD) class of insulin sensitizers, such as rosiglitazone (previously

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known as BRL49653 [BRL]) and pioglitazone,[20] as well as natural ligands, such as the prostaglandin D_2 derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2)[21] and oxidized linoleic acid (9- or 13-HODE).[22] In vitro experiments demonstrate that PPARα and PPARγ activators decrease inflammatory proteins, such as adhesion molecules, cytokines, and chemokines, in monocytes/macrophages, ECs, and vascular smooth muscle cells.[23] Moreover, recent in vivo studies suggest that PPAR activators can limit experimental atherosclerosis in animal models.[24,25] In human trials, preliminary clinical data in diabetic patients suggest that TZD treatment can decrease carotid intimal-medial thickness,[26] and recent studies with PPARα-activating fibric acids have also demonstrated decreased atherosclerosis among treated patients.[27] Interestingly, fenofibrate treatment in patients with coronary heart disease reduced IFNγ plasma levels through an as-yet-undefined mechanism.[28]

Given the role of T-lymphocyte inflammatory cytokine production in atherosclerosis and evidence of PPARs as antiinflammatory mediators, we hypothesized that human T lymphocytes express PPARα and PPARγ and that stimulation of these cells by PPAR activators in clinical use would limit inflammatory cytokine expression. Indeed, concurrent work suggests that PPARγ ligands may influence T-cell activation and proliferation,[29,30] although those studies did not address PPARα in T-cell cytokine responses or PPAR regulation of T-cell IFNγ production.

Materials and Methods

Cell Culture

Human CD4-positive T cells were isolated from freshly drawn blood of healthy volunteers by Ficoll-Histopaque (Sigma Chemical Co) gradient centrifugation to obtain mononuclear cells and subsequent negative selection of CD4-positive T cells by magnetic bead separation (Miltenyi Biotech), as described by the manufacturer's protocol. The purity of CD4-positive T cells was >97%, as determined by flow cytometry. Human ECs and monocytes were isolated as previously described.[31,32]

Reverse Transcriptase–Polymerase Chain Reaction

Total RNA from freshly prepared CD4-positive T cells was isolated for reverse transcriptase (RT)–polymerase chain reaction (PCR) with amplification of PPARα, PPARγ, and GAPDH cDNA as described previously.[15]

Preparation of Nuclear and Cytosolic Extracts and Western Blot Analysis

For Western blotting, nuclear and cytosolic extracts of 10^7 cells were prepared as previously described.[15]

Stimulation Assays, ELISA, and Cell Viability Studies

Human CD4-positive T cells (1×10^6 cells/mL) were pretreated with PPARα activators (WY14643 or fenofibrate) or PPARγ activators (BRL or pioglitazone) for 2 hours before stimulation with immobilized anti-CD3 antibody (R&D Systems) for 48 hours or with phorbol 12-myristate 13-acetate (PMA, 10 ng/mL)/ionomycin (0.5 μmol/L) for 6 hours, according to previously published time courses for these stimuli.[33,34] Cells were then harvested, and IFNγ, TNFα, and IL-2 ELISAs (R&D Systems) were performed on cell-free supernatants, as recommended by the manufacturer. In some experiments, cells were stimulated with PPAR activators for 24 hours, and the release of IL-4, a typical TH2-cytokine, was measured by ELISA (R&D Systems).

Cell viability was assessed by standard trypan blue exclusion, as described previously.

Northern Blot Analysis

For Northern blot experiments, cells were pretreated with PPAR activators and then stimulated with anti-CD3 antibodies for 24 hours or with PMA/ionomycin for 2 hours. Five micrograms of total RNA was used in standard Northern blot analysis by using cDNA probes against IFNγ, TNFα, or IL-2 or against the housekeeping genes B41 or GAPDH.

Flow Cytometry

Immunofluorescence staining and flow cytometry were performed as previously described.[35] Human CD4-positive T cells were incubated with an equal volume of PBS containing saturating concentrations (10 mg/L) of FITC-conjugated anti-CD3 antibodies and PE-conjugated anti-CD4 antibodies for 30 minutes at room temperature. To examine the influence of PPAR activators on the proinflammatory activity of T-cell supernatants toward other vascular cells, freshly isolated human monocytes or human ECs were incubated with supernatants (50% original monocyte or EC media and 50% conditioned media from T cells) derived from T cells after CD3 activation in the absence or presence of WY14643 or BRL (Figures 5A and 6A, right panels). In some experiments, cells were first treated with conditioned media from anti-CD3–activated T cells (Figures 5B and 6B, right panels) or IFNγ (Figures 5C and 6C, right panels), and then PPAR activators at similar concentrations were directly added to monocytes or ECs. After 18 hours (in monocyte experiments) or 72 hours (in EC experiments), cells were harvested for the investigation of monocyte CD64 or endothelial major histocompatibility complex (MHC) class II (MHC II) expression on the cell surface, respectively. After washing, monocytes were stained with FITC-conjugated anti-CD64 antibodies, and ECs were stained with FITC-conjugated anti–MHC II antibodies. Finally, T cells, monocytes, or ECs were washed three times and stored in 1% paraformaldehyde (Sigma) at 4°C until flow cytometric analysis was performed within 24 hours.

Statistical Analysis

Results of the experimental studies are reported as mean±SEM. Differences were analyzed by one-way ANOVA, followed by the appropriate post hoc test. A value of $P<0.05$ was regarded as significant.

Results

Human CD4-Positive T Cells Express PPARα and PPARγ mRNA and Protein

Isolated human CD4-positive T cells express PPARα and PPARγ mRNA as determined by RT-PCR (Figure 1A). Western blot analysis revealed PPARα as well as PPARγ protein expression in the nuclear fraction but not in the cytosol of isolated CD4-positive human T cells (Figure 1B). Induction of IFNγ expression by stimulation with anti-CD3 antibodies and/or treatment with PPAR activators did not affect PPAR expression in these cells (data not shown).

PPAR Activators Inhibit IFNγ Expression in Human CD4-Positive T Cells

Unstimulated human CD4-positive T cells did not secrete IFNγ, as determined by ELISA of cell-free supernatants. As expected, incubation of cells with immobilized anti-CD3 antibodies significantly increased IFNγ protein secretion from 0 to 504±168 pg/mL ($P<0.01$, n=6). Two-hour pretreatment with PPARα activators, either WY14643 or

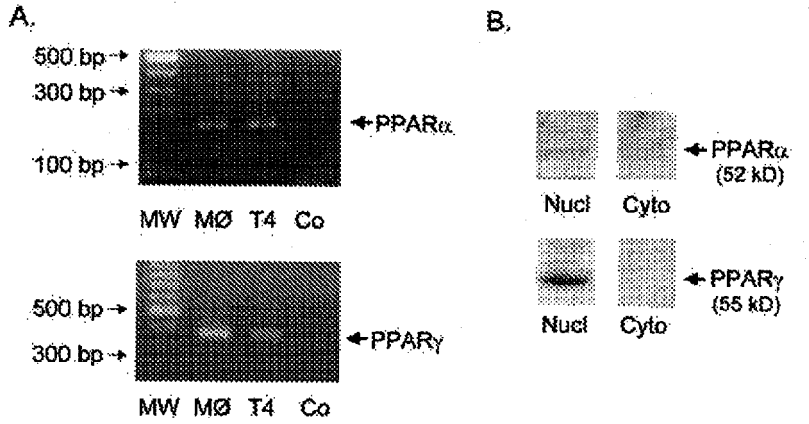

Figure 1. CD4-positive human T cells express PPARα and PPARγ. A, RT-PCR reaction of PPARα and PPARγ RNA in freshly isolated human CD4-positive T cells (T4) reveals a cDNA fragment of the expected size. Also shown are a DNA ladder (MW), RT-PCR product from macrophage RNA as a positive control (MØ), and a negative control consisting of RT-PCR reactions lacking RT (Co). B, Western blot analysis on nucleic (Nucl) and cytosolic (Cyto) fractions of human CD4-positive T cells with use of an anti-human PPARα antibody or an anti-human PPARγ antibody.

fenofibrate, inhibited this increase in a concentration-dependent manner. IFNγ production was not detected at WY14643 (250 μmol/L) and was reduced by fenofibrate to 13±5% of the level elaborated by untreated control cells ($P<0.01$ for both compared with CD3-activated cells without agonist, n=4) (Figure 2A). Similarly, pretreatment of CD4-positive T cells with two different PPARγ-activating TZDs also reduced anti-CD3-induced IFNγ release in a concentration-dependent manner, with a maximal reduction to 52±9% at 10 μmol/L BRL and to 28±8% at 10 μmol/L pioglitazone ($P<0.01$ for both compared with CD3-activated cells, n=6) (Figure 2B). None of the PPAR activators that were used affected cell viability (by trypan blue exclusion) or cell surface CD3 expression, as determined by flow cytometry (Table).

PPAR Activators Reduce the Expression of Other Proinflammatory Cytokines in CD4-Positive Human T Cells

To examine whether the effects of PPAR activators extended beyond IFNγ to other inflammatory cytokines, we performed similar experiments measuring TNFα and IL-2 protein expression of human CD4-positive T cells. Pretreatment of cells with PPARα-activating WY14643 reduced anti-CD3–induced TNFα and IL-2 secretion in a concentration-dependent manner, with maximal inhibition to 7±4% of TNFα production at 250 μmol/L WY14643, and abrogated IL-2 expression under similar conditions ($P<0.01$ for both compared with anti-CD3–activated cells, n=3) (Figure 3A). PPARγ-activating BRL had similar concentration-dependent, albeit less complete, effects; anti-CD3-induced TNFα and IL-2 protein expression decreased to 64±7% and 34±7%, respectively, at 10 μmol/L BRL ($P<0.01$ for both compared with CD3-activated cells, n=3) (Figure 3B). To exclude the possibility that these results stemmed from a shift of T cells toward a TH2 response, we measured IL-4 in supernatants on stimulation with PMA/ionomycin or PPAR activators. PMA/ionomycin treatment induced IL-4 protein secretion from 5±4 to 205±42 pg/mL ($P<0.01$, n=3), whereas none of the PPARα or PPARγ activators had a similar effect (Figure 3C).

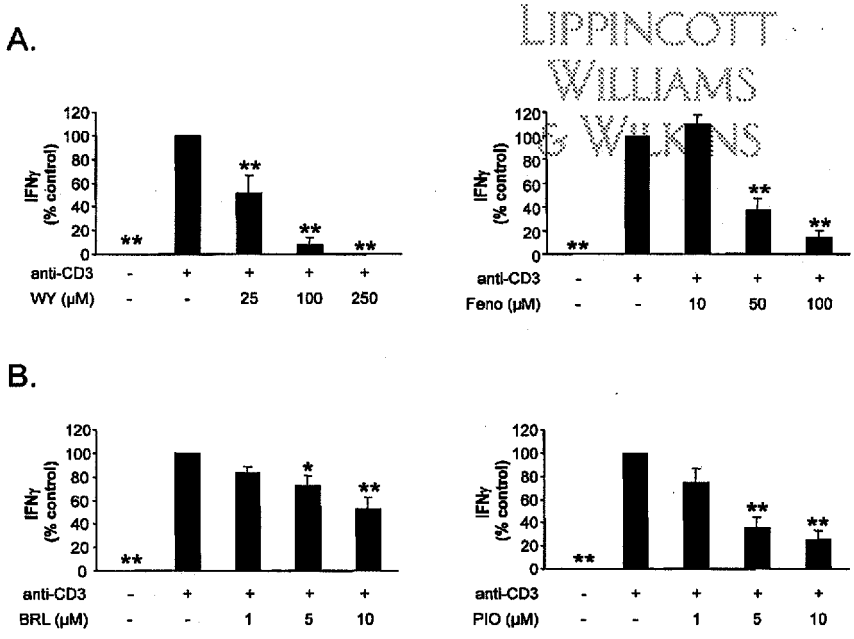

Figure 2. PPAR activators inhibit IFNγ expression in human CD4-positive T cells. A, Isolated CD4-positive T cells were pretreated with PPARα activators (WY14643 or fenofibrate) 2 hours before stimulation with anti-CD3 antibodies. After 48 hours, cytokine protein content in cell-free supernatants was measured by ELISA. Results are expressed as percentage of CD3-activated cells (% control). Bars represent mean±SEM (n=6). $^{**}P<0.01$. B, Isolated CD4-positive T cells were pretreated with PPARγ activators (BRL or pioglitazone) 2 hours before stimulation with anti-CD3 antibodies. After 48 hours, cytokine protein content in cell-free supernatants was measured by ELISA. Results are expressed as percentage of CD3-activated cells (% control). Bars represent mean±SEM (n=6). $^{*}P<0.05$; $^{**}P<0.01$.

Effects of PPAR Activators

	Effects of PPARα Activators on Human CD4-Positive T Cells		
	CD3 Activated	CD3-Activated+ 250 μmol/L WY14643	CD3-Activated+ 100 μmol/L Fenofibrate
Cell viability, %	>90	>90	>90
CD3 expression* (mean±SEM; n=5)	32.0±3.9	29.4±1.0	31.2±1.5
	Effects of PPARγ Activators on Human CD4-Positive T Cells		
	CD3 Activated	CD3-Activated+ 10 μmol/L BRL49653	CD3-Activated+ 10 μmol/L Pioglitazone
Cell viability, %	>90	>90	>90
CD3 expression* (mean±SEM; n=6)	40.3±3.7	37.1±3.8	39.0±3.8

*As determined by flow cytometry.

PPAR Activators Inhibit PMA/Ionomycin-Induced Proinflammatory Cytokine Expression in Human CD4-Positive T Cells

To investigate whether the effects of PPAR activators on T4 cell–derived IFNγ expression depended on the stimulus used, we used PMA/ionomycin to induce IFNγ release. PMA/ionomycin treatment of human CD4-positive T cells stimulated more IFNγ protein expression than did CD3 activation, increasing IFNγ protein content in the supernatant to 4971±1596 pg/mL. Pretreatment of the cells with the PPARα activator WY14643 (250 μmol/L) reduced IFNγ release to 36±10% ($P<0.01$ compared with PMA/ionomycin-stimulated cells, n=3), whereas pretreatment with the PPARγ activator BRL decreased IFNγ-protein secretion to 71±3% ($P<0.05$ compared with PMA/ionomycin-stimulated cells, n=3) (Figure 3D).

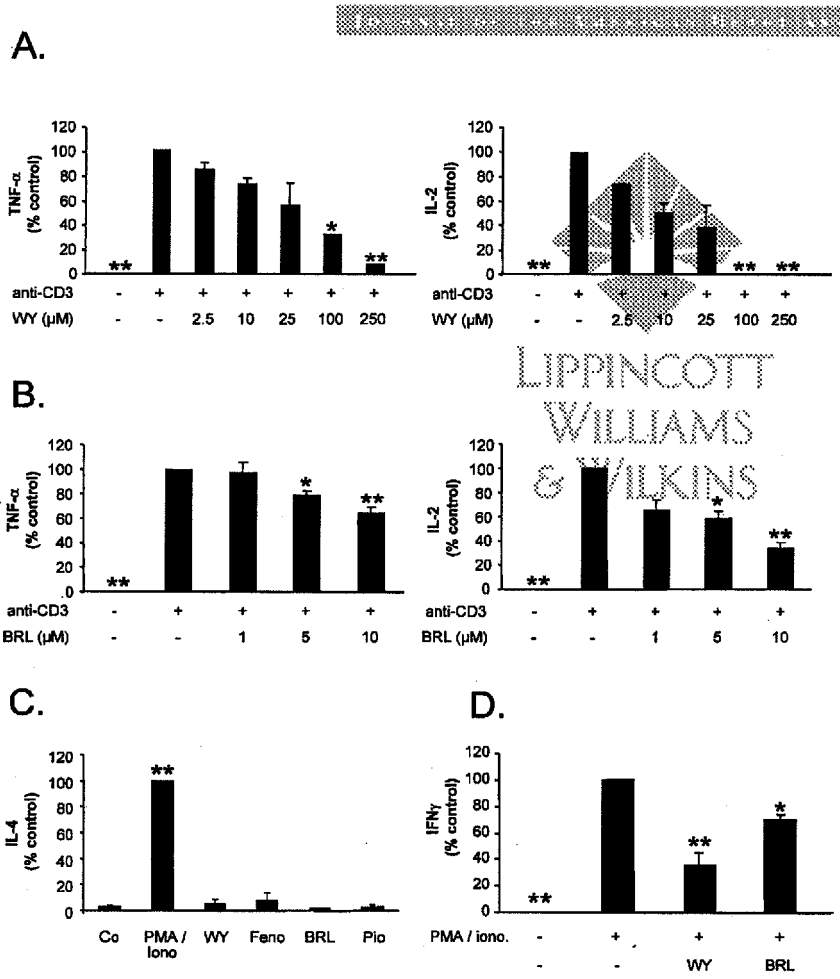

Figure 3. PPAR activators reduce TNFα and IL-2 protein secretion from CD4-positive human T cells. A and B, Isolated CD4-positive T cells were pretreated with PPARα-activating WY14643 (A) or PPARγ-activating BRL 2 hours before stimulation with anti-CD3 antibodies (B). After 48 hours, cytokine protein content in cell-free supernatants was measured by ELISA. Results are expressed as percentage of CD3-activated cells (% control). Bars represent mean±SEM (n=3). *$P<0.05$; **$P<0.01$. C, PPAR activators do not induce expression of IL-4 in CD4-positive human T cells. Isolated CD4-positive T cells were treated with PPARα activators (250 μmol/L WY14643 or 100 μmol/L fenofibrate) or PPARγ activators (10 μmol/L BRL or 10 μmol/L pioglitazone) for 24 hours before IL-4 protein content in cell-free supernatant was measured by ELISA. PMA/ionomycin (100 ng/mL/1 μmol/L)–treated cells served as a positive control. Results are expressed as percentage of PMA/ionomycin-activated cells (% control). Bars represent mean±SEM (n=3). **$P<0.01$ compared with unstimulated cells. D, PPAR activators inhibit PMA/ionomycin-induced IFNγ expression. Isolated CD4-positive T cells were stimulated and treated with PMA/ionomycin (10 ng/mL/0.5 μmol/L) in the absence or presence of the PPARα activator WY14643 or the PPARγ activator BRL. After 6 hours, cytokine protein contents in cell-free supernatants were measured by ELISA. Results are expressed as percentage of PMA/ionomycin-treated cells (% control). Bars represent mean±SEM (n=3). *$P<0.05$; **$P<0.01$.

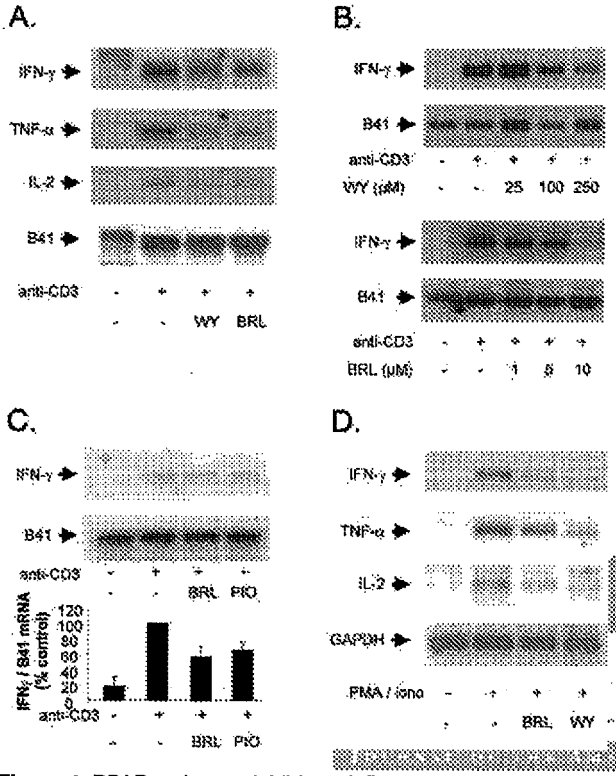

Figure 4. PPAR activators inhibit proinflammatory cytokine mRNA expression in human CD4-positive cells. A, Representative Northern blot analysis for cytokine expression of human CD4-positive T cells pretreated with WY14643 (250 μmol/L) or BRL (10 μmol/L) before 24-hour stimulation with anti-CD3 antibodies. Three independent experiments yielded similar results. B, Representative Northern blot analysis for cytokine expression of human CD4-positive T cells pretreated with WY14643 (top) or BRL (bottom) at concentrations indicated before incubation with anti-CD3 antibodies for 24 hours. Three independent experiments yielded similar results. C, Representative Northern blot analysis for cytokine expression of human CD4-positive T cells pretreated with BRL or pioglitazone (both at 10 μmol/L) before incubation with anti-CD3 antibodies for 24 hours (top). At the bottom is a densitometric analysis of IFNγ mRNA expression normalized to housekeeping gene B41 of 3 independent experiments. D, Representative Northern blot analysis for cytokine mRNA expression of human CD4-positive T cells pretreated with WY14643 (250 μmol/L) or BRL (10 μmol/L) before 2-hour stimulation with PMA/ionomycin. Three independent experiments yielded similar results.

PPAR Activation Reduces Cytokine mRNA Expression in Human T4 Cells

To examine whether the decrease in proinflammatory cytokine expression by PPAR activators resulted from reduced cytokine mRNA expression, we pretreated CD4-positive T cells with PPARα or PPARγ activators and performed Northern blot analysis after 24-hour stimulation with anti-CD3 antibodies. PPARα-activating WY14643 or PPARγ-activating BRL markedly reduced anti-CD3–induced IFNγ, TNFα, and IL-2 mRNA content but did not affect mRNA levels of the constitutively expressed gene B41 (Figure 4A). The inhibition of anti-CD3–induced IFNγ mRNA expression by WY14643 or BRL was concentration dependent, as shown in Figure 4B. In contrast to the results on protein expression, BRL or pioglitazone produced similar inhibition of IFNγ mRNA expression, as determined by densitometry of three different Northern blots (Figure 4C). In addition, the effects observed were not dependent on the stimulus used, as shown by similar WY14643 and BRL effects on PMA/ionomycin-induced IFNγ, TNFα, and IL-2 mRNA (Figure 4D).

PPAR Activators Reduce Proinflammatory Function of T Lymphocytes on Human Monocytes and ECs

To examine the potential functional effects of PPAR-mediated reduced T-cell cytokine expression, we incubated supernatants from stimulated CD4-positive T cells with human monocytes or ECs and measured monocyte CD64 or endothelial MHC II surface expression by flow cytometry. CD64, the high-affinity receptor for IgG involved in phagocytosis and antigen capture, an IFNγ-regulated gene in human monocytes, indicates IFNγ activity on monocytes in vitro and in vivo.[36] In addition, IFNγ potently stimulates MHC II expression on ECs and acts synergistically with TNFα. Incubation of freshly isolated human monocytes with supernatants from CD3-activated T cells significantly increased monocyte CD64 cell surface expression by ≈2-fold. Supernatants taken from activated CD4-positive T cells after WY14643 or BRL treatment reduced this increase significantly to 61±8% or 72±4%, respectively ($P<0.01$ or $P<0.05$, respectively, compared with monocytes incubated with supernatant from CD3-activated T cells; n=4) (Figure 5A), consistent with reduced cytokine content in the media (data not shown). To exclude the possibility that the results observed resulted from direct effects of residual PPAR agonist in T-cell supernatants, we stimulated human monocytes with conditioned media from CD4-positive cells to induce CD64 expression and then added WY14643 or BRL directly to the cells. None of the PPAR activators used had direct significant effects on monocyte CD64 expression (Figure 5B). Consistent with this finding, PPAR activators did not affect IFNγ-induced CD64 expression in human monocytes (Figure 5C).

Mean fluorescence intensity of MHC II expression in human ECs incubated with supernatants from unstimulated CD4-positive T cells was 10±4 (arbitrary units). Incubation of ECs with supernatants taken from CD3-activated T cells significantly increased MHC II cell surface expression to 51±13 ($P<0.05$, n=5). Medium conditioned by activated CD4-positive T cells after WY14643 or BRL treatment showed significantly reduced MHC II expression (64±17% or 53±14%, respectively; $P<0.05$ compared with ECs incubated with supernatant from CD3-activated T cells; n=5) (Figure 6A). Neither WY14643 nor BRL directly affected T-cell media– or IFNγ-induced endothelial MHC II expression (Figures 6B and 6C).

Discussion

The present study reports PPARα and PPARγ expression in human CD4-positive T cells with evidence of inhibition of inflammatory cytokine production by PPARα-activating fibric acid derivatives or PPARγ-activating TZDs in these cells. These results have potential physiological significance, given our finding that monocytes and ECs demonstrate

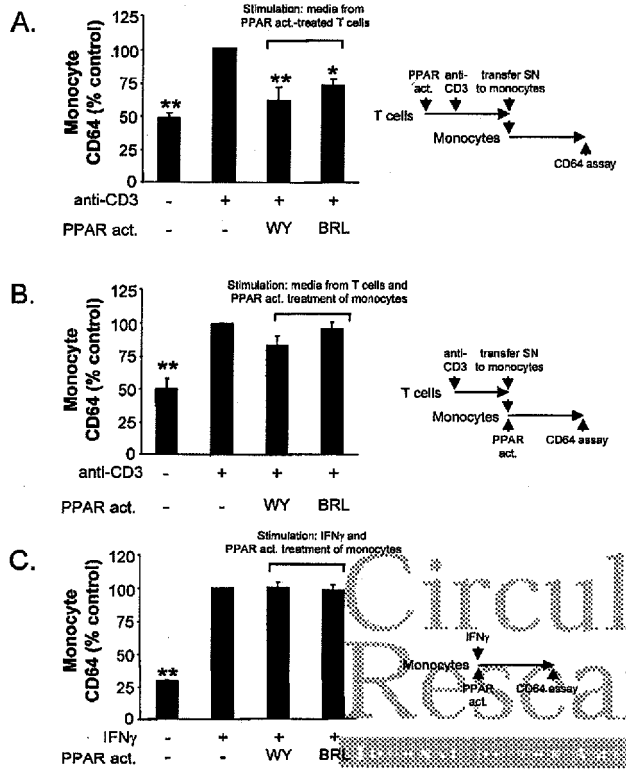

Figure 5. PPAR activators reduce proinflammatory activity of T-cell supernatants on human monocytes in the absence of any direct effects on monocyte CD64 response. A, Freshly isolated human monocytes were incubated for 18 hours with conditioned media from CD4-positive T cells stimulated with anti-CD3 monoclonal antibodies in the presence or absence of PPAR agonists, and mean fluorescence intensity of monocyte CD64 expression was measured by flow cytometry (right). Results are expressed as percentage of control (monocytes incubated with supernatants from activated T cells). Bars represent mean±SEM (n=4). *$P<0.05$; **$P<0.01$. B, Human monocytes were incubated with conditioned media from CD3-activated T cells to induce CD64 expression and then directly stimulated with PPAR activators for 18 hours before CD64 expression was assessed by flow cytometry (right). Results are expressed as percentage of control (monocytes incubated with supernatants from activated T cells). Bars represent mean±SEM (n=4). No significant difference was seen, except for comparison with unstimulated cells. **$P<0.01$. C, Human monocytes were incubated with IFNγ (200 U/L) in the presence or absence of PPAR activators. After 18 hours, CD64 expression was measured by flow cytometry (right). Results are expressed as percentage of control (monocytes stimulated with IFNγ). Bars represent mean±SEM (n=5). No significant difference was seen, except for comparison with unstimulated cells. **$P<0.01$.

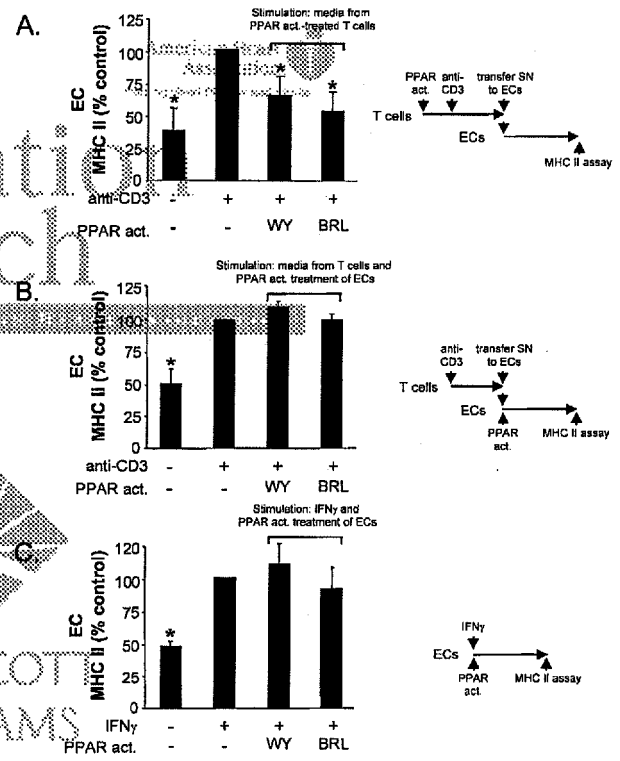

Figure 6. PPAR activators reduce proinflammatory activity of T-cell supernatants on human ECs. A, Human ECs were incubated for 72 hours with conditioned media from CD4-positive T cells stimulated with or without PPAR activators, and endothelial MHC II surface expression was measured by flow cytometry (mean fluorescence intensity) (right). Results are expressed as percentage of ECs incubated with supernatants from activated T cells. Bars represent mean±SEM (n=3). *$P<0.05$. B, Human ECs were incubated with conditioned media from CD3-activated T cells to induce MHC II expression and then directly stimulated with PPAR activators for 72 hours before MHC II surface expression was assessed by flow cytometry (right). Results are expressed as percentage of control (ECs incubated with supernatants from activated T cells). Bars represent mean±SEM (n=4). No significant difference was seen, except for comparison with unstimulated cells. **$P<0.01$. C, Human ECs were incubated with IFNγ (1000 U/L) in the presence or absence of PPAR activators. After 72 hours, MHC II expression was measured by flow cytometry (right). Results are expressed as percentage of control (ECs stimulated with IFNγ). Bars represent mean±SEM (n=4). No significant difference was seen, except for comparison with unstimulated cells. *$P<0.05$.

reduced responses toward the proinflammatory effects of activated T cells treated with these same PPAR activators.

Although PPAR expression was initially considered to be restricted to tissues like liver and fat, recent work has demonstrated PPARα and PPARγ expression in vascular cells, such as monocytes/macrophages, ECs, and smooth muscle cells.[23] Recent studies also documented PPARγ expression in murine and human T lymphocytes.[29,30] Previous work has not addressed PPARα expression by lymphocytes. The decrease in IFNγ expression described in the present study likely occurred through the activation of PPARα and PPARγ by their respective agonists, given that such concentrations are similar to those found in the plasma of patients treated with these agonists.[37] However, the results shown in the present study do not conclusively establish that the effects were due to specific receptor activation. Interestingly, recent work has revealed that some effects of TZDs could occur independent of the presence of PPARγ, at least in cells of the monocytic lineage.[38] Although monocyte/macrophage responses differ in substantive ways from T-cell responses, particularly in terms of cytokine induction, the intriguing possibility that some of the effects observed in the present study might be PPARγ independent cannot be excluded. Regardless, these data reveal novel effects of antidiabetic TZDs on T lymphocytes and their interaction with vascular

cells, with potential clinical relevance for patients. Interestingly, pioglitazone, despite a lower binding affinity to PPARγ, was more potent than BRL in inhibiting IFNγ protein production. Our results with PPARα agonists suggest that this might be due to a combined PPARα and PPARγ effect of pioglitazone, given that this agent (in contrast to BRL) can also activate PPARα.[39] The lack of a difference between pioglitazone and BRL on mRNA expression and the mild suppression of cytokine mRNA compared with protein levels suggest that posttranscriptional modification may also play a role. In this regard, recent work has shown that TZDs inhibit the initiation of translation independent of PPARγ,[40] and similar mechanisms may be at work in our findings.

The effects of PPARα and PPARγ activators on human T cells extend to inhibition of other proinflammatory cytokines, including TNFα and IL-2, implicating PPARs as a potential nodal point for the regulation of T-cell–modulated inflammatory responses. In addition, the results obtained do not derive from a shift of T cells toward a TH2 response, because none of the PPAR activators used increased the levels of IL-4, a classic TH2 cytokine, in CD4-positive T cells.

Prior reports demonstrating the effects of PPARγ agonists on lymphocytes varied from ours in design and results in important ways. These studies used PPAR agonists at higher concentrations (TZDs at 20 to 40 μmol/L), which are thought unlikely to prevail in vivo, or the studies used T-cell lines rather than primary isolates.[30] Clark et al[30] found reduced IL-2 secretion from murine T-cell clones after treatment with the PPARγ activator ciglitazone (20 to 40 μmol/L) and the putative PPARγ agonist, but they did not examine the effect on IFNγ and TNFα. These higher concentrations raise the potential for pleiotropic effects, toxicity, and increased cross-reaction with other nuclear receptors. Yang et al[29] showed that the PPARγ activators troglitazone and 15d-PGJ$_2$ decrease IL-2 production in human T cells, whereas the PPARα activator WY14643 had no effects on phytohemagglutinin/PMA-induced IL-2 release. Beyond issues specific to each agonist, eg, the potential antioxidant properties of troglitazone or the low concentrations used for WY14643, relevant experimental differences include the use of mixed T-lymphocyte populations as opposed to selected CD4-positive cells, the nature of the stimuli used to induce IL-2 expression, and the differing protocols for the addition of agonists (concurrent addition versus pretreatment). Harris and Phipps[41] recently found PPARγ expression in a transgenic lymphocyte mouse cell line (D011.10) and induction of apoptosis by troglitazone and 15d-PGJ$_2$ at high concentrations (10 to 100 μmol/L).

We find that stimulation of isolated CD4-positive human T cells, when stimulated with canonical PPARα and PPARγ agonists at clinically relevant concentrations, demonstrates decreased IFNγ, TNFα, and IL-2 production, with no effect on viability. Such findings likely have relevance to the function of T lymphocytes in atherosclerosis and Tx-AA. In human atheroma, activated CD4-positive T cells release inflammatory cytokines such as IFNγ, TNFα, and IL-2, presumably promoting lesion progression through the activation of other vascular cells in a paracrine fashion.[4] In ECs, these T-cell–derived cytokines induce the expression of leukocyte-recruiting chemokines, such as monocyte chemoattractant protein-1 or interferon-inducible protein of 10 kDa,[42] and the expression of adhesion molecules. Such actions may contribute to an ongoing cycle of inflammatory cell recruitment, attachment, and migration into the vessel wall, along with further cellular activation. Similar inflammatory effects contribute to Tx-AA, a condition in which IFNγ-induced MHC class II expression on the surface of donor ECs triggers host T-cell activation.[10] A reduction of IFNγ release with inhibition of endothelial MHC class II expression, as shown in the present study, raises the possibility that PPAR agonists might modulate allograft vasculopathy.

In monocytes/macrophages, IFNγ stimulates the secretion of cytokines,[6] whereas in smooth muscle cells, IFNγ inhibits proliferation and extracellular matrix synthesis.[5] This mechanism might destabilize the protective fibrous cap of the lesion and, thus, contribute to plaque rupture with its sequelae, such as unstable angina or acute myocardial infarction. Interestingly, patients with unstable angina show increased IFNγ production by CD4-positive cells,[8] bolstering the hypothesis that T-cell activation contributes to the acute coronary syndromes. In contrast, Tx-AA is characterized by smooth muscle cell proliferation, which is thought to be driven in part by cytokine and cytokine-induced growth factors. PPARγ agonists may oppose this response. The antiinflammatory effects of PPAR agonists on T lymphocytes presented in the present study or their reported effects on other gene targets in mononuclear or vascular wall cells might contribute to decreased cardiovascular events or Tx-AA in patients. Although it remains impossible to establish that the clinical effects of these agents occur through PPAR activation, noteworthy recent clinical trials of fibrates have shown decreases in atherosclerosis[27] and cardiovascular events.[43] PPARγ agonists have shown benefits in surrogate cardiovascular end points, such as carotid intimal-medial thickness and restenosis in humans.[44] With increasing evidence of inflammatory pathways not only in atherosclerosis but also in the development of diabetes itself,[45] the results reported in the present study suggest that PPAR modulation of inflammatory pathways in T cells may offer clinical benefits in pathological processes, such as atherosclerosis and Tx-AA, and is certainly worthy of study in future clinical trials with PPAR agonists.

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(54) Title: ACTIVATORS FOR PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR

(54) 発明の名称: ペルオキシゾーム増殖剤活性化受容体の活性化剤

(57) Abstract: Activators for peroxisome proliferator-activated receptor which contain polyprenyl compounds, preferably (2E,4E,6E,10E)-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid as the active ingredient; and drugs for preventing and/or treating hyperlipidemia, non-insulin dependent diabetes mellitus, etc. which contain, as the active ingredient, polyprenyl compounds.

(57) 要約:

ポリプレニル化合物、好ましくは(2E, 4E, 6E, 10E)-3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸を有効成分として含むペルオキシゾーム増殖剤活性化受容体の活性化剤、及びポリプレニル化合物を有効成分として含む高脂血症又はインスリン非依存性糖尿病等の予防及び／又は治療のための医薬。

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明 細 書

ペルオキシゾーム増殖剤活性化受容体の活性化剤

技術分野

本発明は、ペルオキシゾーム増殖剤活性化受容体(peroxisome proliferator-activated receptor：以下、本明細書において「PPAR」と略す場合がある)の活性化剤に関するものである。

背景技術

現在、我が国の高脂血症患者及び糖尿病患者は併せて1000万人以上と推定され、その数は増加の一途にある。糖尿病患者の多くはインスリン非依存性糖尿病であり、その特徴はインスリンの作用に抵抗性を示す高血糖等を呈する病態である。また、高脂血症や糖尿病では、高インスリン血症、低HDLコレステロール血症、高血圧及び肥満等の病態が高頻度に発生し、臨床上問題とされている。近年、これら複数の症状を示す病態はX症候群と呼ばれ、重篤な疾患として位置付けられている（引用文献：Diabetes、37、1595-1607(1988)）。

これらの疾患に対する治療薬として、クロフィブレートに代表されるクロフィブレート系誘導体やピオグリタゾン、トログリタゾンに代表されるチアゾリジン誘導体等が使用されている。クロフィブレート系誘導体はPPARαの活性化作用を有し（引用文献：Nature、347、645-650(1990)）、肝臓において脂肪酸β酸化系酵素を介して脂質代謝を改善すると考えられている。また、チアゾリジン誘導体はPPARγの活性化作用を有し(引用文献:J. Biol. Chem.、270、112953-112956(1995))、インスリン抵抗性を改善し血糖を低下させると考えられている（引用文献：Diabetes、45、1661-1669(1996)）。

しかしながら、PPARの作動薬は一般的に肝機能障害等の副作用が報告されており、PPARγ作用薬の一つであるトログリタゾンは肝機能障害患者には禁忌であり

(引用文献：臨床医薬、14、461-466(1998))、現在は販売中止となっている。

上記のように、PPARの活性化作用を有する薬物は高脂血症及び糖尿病に対する治療薬として有用であるが、副作用が多いことから副作用の少ないPPARの活性化薬が望まれている。

ポリプレニル化合物の一つである(2E, 4E, 6E, 10E)-3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸（開発コード「NIK-333」）は、レチノイン酸結合蛋白及びレチノイン酸受容体に対して親和性を示すことや、肝細胞癌における分化誘導作用及びアポトーシス誘導作用が知られている。臨床においては、NIK-333は一年間の長期投与により肝癌根治治療後の再発を有意に抑制し、肝癌再発抑制作用が示唆されている。さらに、この時、肝機能障害及び他のレチノイドに見られる副作用はほとんど認められず、安全な薬剤である（引用文献：N. Eng. J. Med. 334、1561-1567 (1996))。

しかしながら、ポリプレニル化合物がPPARを活性化することは全く知られていない。

発明の開示

従って、本発明は副作用の少ないPPARの活性化剤を提供することを目的とするものである。

本発明者らは、PPARの活性化剤を見出すべく種々研究を重ねてきた。その結果、ポリプレニル化合物がPPARα及びγのmRNA発現を誘導し、さらにPPARαに対してリガンド活性を有することを明らかにした。これらの結果から、ポリプレニル化合物はPPARを活性化することを見出し、さらに研究を行うことにより本発明を完成するに至った。

すなわち、本発明は、ポリプレニル化合物を有効成分とするペルオキシゾーム増殖剤活性化受容体(PPAR)の活性化剤を提供するものである。また、本発明により、ポリプレニル化合物を有効成分として含む高脂血症又はインスリン非依存性糖尿病の予防及び／又は治療のための医薬が提供される。

別の観点からは、上記の医薬の製造のためのポリプレニル化合物の使用；ヒトを含む哺乳類動物においてペルオキシゾーム増殖剤活性化受容体(PPAR)を活性化する方法であって、ポリプレニル化合物の有効量をヒトを含む哺乳類動物に投与する工程を含む方法；並びに、高脂血症又はインスリン非依存性糖尿病の予防及び／又は治療方法であって、予防及び／又は治療を必要とするヒトを含む哺乳類動物にポリプレニル化合物の予防及び／又は治療有効量を投与する工程を含む方法が本発明により提供される。

図面の簡単な説明

第１図は、NIK-333 で処理した細胞中の PPARαの mRNA (wild type、splice variant) の発現を示す。

第２図は、NIK-333 で処理した細胞中の PPARγ１の mRNA の発現を示す。

第３図は、NIK-333 又は Wy-14643 の PPARαの発現ベクター未導入時（－）及び導入時（＋）における PPARα に対するリガンド活性を示す。

発明を実施するための最良の形態

日本国特願 2000-122974 号（2000 年 4 月 24 日出願）の明細書の全ての開示を本明細書の開示に参照として含める。

本発明に使用されるポリプレニル化合物としては、(2E, 4E, 6E, 10E)-3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸 (NIK-333) を特に好ましい化合物として挙げることができる。また、他のポリプレニル化合物として、特公昭 63-34855 公報に記載の 3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸等の共役ポリプレニルカルボン酸（ポリプレン酸）及びそのエステルなどを挙げることができる。

本発明で使用されるポリプレニル化合物は、公知の方法（日本国特許公報昭 63-32058 号、J. Chem. Soc. (C), 2154 頁, 1966 年）により合成することができる。

本発明のPPAR活性化剤、あるいはPPARの活性化作用に基づく本発明の高脂血症又はインスリン非依存性糖尿病の予防及び／又は治療のための医薬を使用する場合には、通常、ポリプレニル化合物を含む医薬組成物を調製し、経口又は非経口のいずれか適当な投与方法により投与することができる。経口投与に適する医薬組成物の形態としては、例えば錠剤、顆粒剤、カプセル剤、軟カプセル剤、丸剤、散剤、液剤などが挙げられ、非経口投与に適する医薬組成物の形態としては、例えば、注射剤、座剤などが挙げられる。これらの医薬組成物は、ポリプレニル化合物又は薬理学上許容しうるその塩と薬学的に許容される通常の製剤担体の１種又は２種以上とを用いて常法により調製することができる。有効成分であるポリプレニル化合物を２種以上用いてもよい。

例えば、経口投与に適する医薬の場合には、製剤担体として、乳糖、ブドウ糖、コーンスターチ、ショ糖などの賦形剤、カルボキシメチルセルロースカルシウム、ヒドロキシプロピルセルロースなどの崩壊剤、ステアリン酸カルシウム、ステアリン酸マグネシウム、タルク、ポリエチレングリコール、硬化油などの滑沢剤、ヒドロキシプロピルセルロース、ヒドロキシプロピルメチルセルロース、カルボキシメチルセルロース、ポリビニルアルコール、ゼラチン、アラビアゴムなどの結合剤、グリセリン、エチレングリコールなどの湿潤剤、その他必要に応じて界面活性剤、矯味剤などを使用して所望の医薬組成物を調製することができる。

また、非経口投与に適する医薬の場合には、製剤担体として、水、エタノール、グリセリン、プロピレングリコール、ポリエチレングリコール、植物油、寒天、トラガラントガムなどの希釈剤を用いて、必要に応じて溶解補助剤、懸濁化剤、乳化剤、安定剤、緩衝剤、等張化剤、保存剤、無痛化剤などを使用することができる。

本発明の医薬は、PPARの活性化により治療及び／又は予防が可能な疾患に適用することができ、ヒトを含む哺乳類動物に対して用いることが可能である。本発明の医薬により活性化可能なPPARとしては、例えばPPARα又はPPARγを好ましい対象として挙げることができる。本発明の医薬の好ましい適用対象としては、

インスリン非依存性糖尿病又は高脂血症、あるいはそれらの疾患の合併症、例えば高インスリン血症、低ＨＤＬコレステロール血症、高血圧、肥満等を挙げることができる。

本発明のPPAR活性化剤、あるいはPPARの活性化作用に基づく本発明の高脂血症又はインスリン非依存性糖尿病の予防及び／又は治療のための医薬を用いる場合、その投与量は特に限定されないが、例えば、成人１日あたり経口投与の場合には１～２，０００ｍｇ、好ましくは２０～８００ｍｇ、非経口投与の場合には１～１，０００ｍｇ、好ましくは１０～１００ｍｇの範囲である。上記の投与量をそれぞれ１日１～３回投与することにより所望の予防及び／又は治療効果が期待できる。

実施例

以下に実施例を挙げて本発明をさらに詳細に説明するが、本発明はこれらの実施例に限定されるものではない。

実施例１　ヒト細胞株でのPPARα及びPPARγのmRNAの発現

ヒト細胞株としてCaco-2（大腸癌由来）を、10%牛胎仔血清を含むRPMI-1640培地にて、37℃で5%CO_2存在下で培養した。その後、無血清のRPMI-1640培地に培地を交換し48時間培養した後、NIK-333の作用を検討するため、NIK-333のエタノール溶液を最終濃度10μMになるように添加した。添加0、0.5、1、2、5時間後にRNAを抽出し、RT-PCR法により、PPARα、PPARγ１及びPPARγ２のmRNAを観察した。

その結果、NIK-333は添加0.5時間後よりPPARαのmRNAの発現が認められた（第１図）。また、NIK-333はPPARγ１のmRNAに対しても添加0.5時間後より発現が認められたが（第２図）、PPARγ２のmRNAの発現は認められなかった。

実施例２　PPARαに対するリガンド活性

サル腎由来細胞株COS-7を、10%牛胎仔血清を含むDMEM培地にて、37℃で5%

CO_2 存在下で培養した。その後、RXRα（レチノイン酸X受容体α）とPPARαの発現ベクター及びその応答配列であるPPRE（ペルオキシゾーム増殖剤応答配列）をくみこんだレポーターベクターを細胞内に同時に導入し24時間培養し、NIK-333の作用を検討するため、NIK-333又はWy-14643（PPARαの選択的作用薬）のエタノール溶液を最終濃度10μMになるように添加した。その24時間培養後、ホタルルシフェラーゼの活性を測定した。測定値はウミシイタケルシフェラーゼ活性で標準化した値で示した。

第3図に示すように、NIK-333及びWy-14643はPPARαの発現ベクター未導入時（－）ではルシフェラーゼ活性を増加せず、PPARαの発現ベクター導入時（＋）のみルシフェラーゼ活性を増加させた。このNIK-333の作用は、Wy-14643と同等のリガンド活性の増加作用を示した。

産業上の利用可能性

ポリプレニル化合物はPPARα及びγの発現を誘導し、さらにPPARαに対するリガンド活性を有することから、PPARの活性化作用を有しており、高脂血症又はインスリン非依存性糖尿病の予防及び／又は治療に有用である。

請求の範囲

1．ポリプレニル化合物を有効成分として含むペルオキシゾーム増殖剤活性化受容体の活性化剤。

2．ポリプレニル化合物がポリプレニルカルボン酸である請求の範囲第1項に記載の活性化剤。

3．ポリプレニル化合物が 3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸である請求の範囲第1項に記載の活性化剤。

4．ポリプレニル化合物が(2E, 4E, 6E, 10E)-3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸である請求の範囲第1項に記載の活性化剤。

5．さらに薬学的に許容される製剤担体を含む医薬組成物の形態である請求の範囲第1項から第4項のいずれか1項に記載の活性化剤。

6．経口投与可能な形態である請求の範囲第1項から第5項のいずれか1項に記載の活性化剤。

7．ペルオキシゾーム増殖剤活性化受容体がPPARα又はPPARγである請求の範囲第1項から第6項のいずれか1項に記載の活性剤。

8．ポリプレニル化合物を有効成分として含む高脂血症又はインスリン非依存性糖尿病の予防及び／又は治療のための医薬。

9．ポリプレニル化合物がポリプレニルカルボン酸である請求の範囲第8項に記載の医薬。

10．ポリプレニル化合物が 3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸である請求の範囲第8項に記載の医薬。

11．ポリプレニル化合物が(2E, 4E, 6E, 10E)-3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸である請求の範囲第8項に記載の医薬。

12．薬学的に許容される製剤担体をさらに含む医薬組成物の形態の請求の範囲第8項から第11項のいずれか1項に記載の医薬。

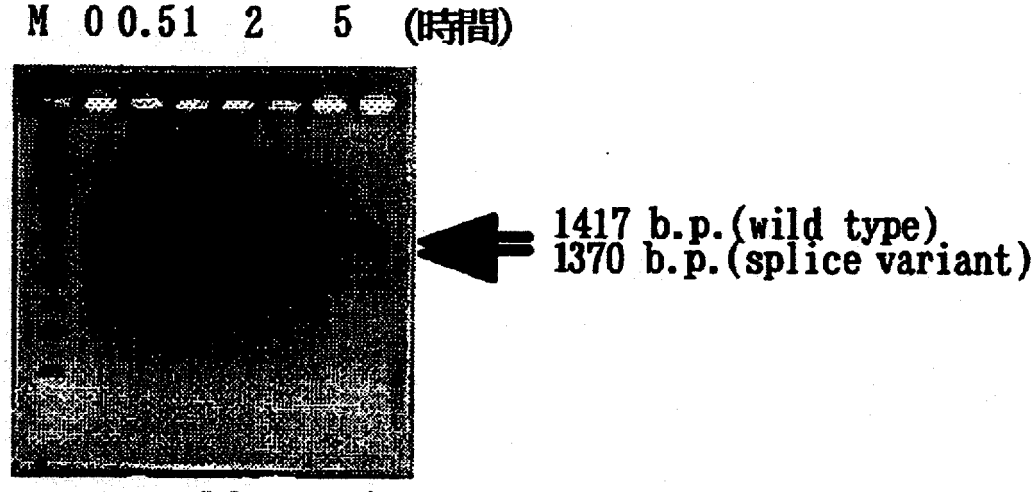
13．経口投与可能な形態である請求の範囲第8項から第12項のいずれか1項

に記載の医薬。

要約書

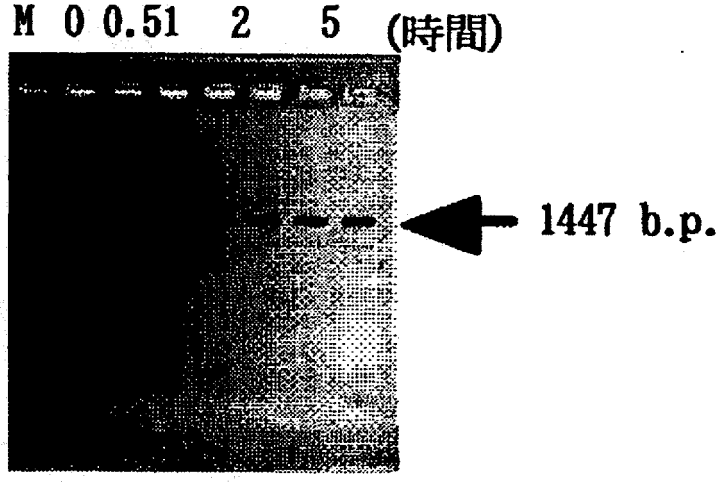
ポリプレニル化合物、好ましくは(2E, 4E, 6E, 10E)-3, 7, 11, 15-テトラメチル-2, 4, 6, 10, 14-ヘキサデカペンタエン酸を有効成分として含むペルオキシゾーム増殖剤活性化受容体の活性化剤、及びポリプレニル化合物を有効成分として含む高脂血症又はインスリン非依存性糖尿病等の予防及び／又は治療のための医薬。

第１図



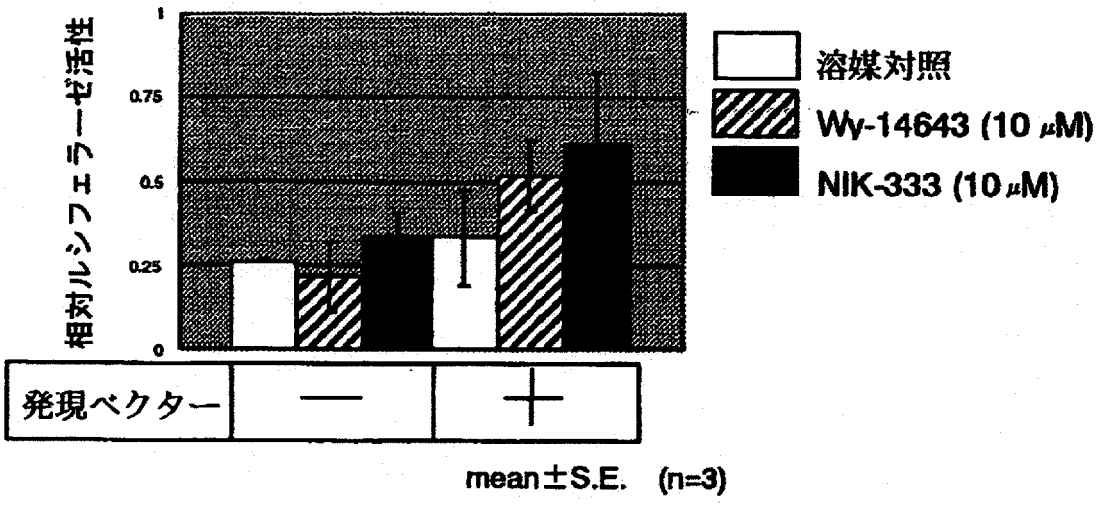
M：マーカー

第２図



M：マーカー

第３図



mean±S.E. (n=3)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/03442

A. CLASSIFICATION OF SUBJECT MATTER
Int.Cl[7] A61K31/202, A61P3/06, 3/10, 43/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
Int.Cl[7] A61K31/202, A61P3/06, 3/10, 43/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
CAPLUS (STN), MEDLINE (STN), EMBASE (STN)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category°	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X Y	ELLINGHAUS, Peter, et al., "Phytanic acid activates the peroxisome proliferator-activated receptor α (PPARα) in sterol carrier protein 2-/sterol carrier protein x-deficient mice", J. Biol. Chem., (1999), Vol.274, No.5, pages 2766 to 2772 & Database CAPLUS on STN, American Chemical Society (ACS), (Columbus, OH, USA), DN.130:265265	1,2,5-9,12,13 1-13
Y	KLIEWER, Steven A., et al., "Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ", Proc. Natl. Acad. Sci. U.S.A., (1997), Vol.94, No.9, pages 4318 to 4323 & Database CAPLUS on STN, American Chemical Society (ACS), (Columbus, OH, USA), DN.127:30658	1-13

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

° Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
04 July, 2001 (04.07.01)	17 July, 2001 (17.07.01)
Name and mailing address of the ISA/ Japanese Patent Office Facsimile No.	Authorized officer Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP01/03442

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	GOETTLICHER, Martin, et al., "Structural and metabolic requirements for activators of the peroxisome proliferator-activated receptor", Biochem. Pharmacol., (1993), Vol.46, No.12, pages 2177 to 2184 & Database CAPLUS on STN, American Chemical Society (ACS), (Columbus, OH, USA), DN.120:159789	1-13
Y	ISSEMANN, I., et al., "The peroxisome proliferator-activated receptor: retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipemic drugs", J. Mol. Endocrinol., (1993), Vol.11, No.1, pages 37 to47 & Database CAPLUS on STN, American Chemical Society (ACS), (Columbus, OH, USA), DN.119:263421	1-13
X Y	EP 194693 A1 (Eisai Co., Ltd.), 17 September, 1996 (17.09.96), & US 4788330 A & US, 4883916 A & JP 61-210050 A	8,9,12,13 8-13
Y	EP 54732 A1 (Eisai Co., Ltd.), 30 June, 1982 (30.06.82), & US 4655973 A & JP 57-106638 A	8-13

国際調査報告

国際出願番号 PCT/JP01/03442

A. 発明の属する分野の分類（国際特許分類（IPC））

Int. Cl' A61K31/202, A61P3/06, 3/10, 43/00

B. 調査を行った分野

調査を行った最小限資料（国際特許分類（IPC））

Int. Cl' A61K31/202, A61P3/06, 3/10, 43/00

最小限資料以外の資料で調査を行った分野に含まれるもの

国際調査で使用した電子データベース（データベースの名称、調査に使用した用語）

CAPLUS(STN), MEDLINE(STN), EMBASE(STN)

C. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
X	ELLINGHAUS, Peter, et al. Phytanic acid activates the peroxisome proliferator-activated receptor α (PPARα) in sterol carrier protein 2-/sterol carrier protein x-deficient mice. J. Biol. Chem., 1999, Vol.274, No.5, pages 2766 to 2772	1,2,5-9,12,13
Y	& Database CAPLUS on STN, AMERICAN CHEMICAL SOCIETY (ACS), (Columbus, OH, USA), DN.130:265265	1-13

☒ C欄の続きにも文献が列挙されている。　☐ パテントファミリーに関する別紙を参照。

* 引用文献のカテゴリー
「A」特に関連のある文献ではなく、一般的技術水準を示すもの
「E」国際出願日前の出願または特許であるが、国際出願日以後に公表されたもの
「L」優先権主張に疑義を提起する文献又は他の文献の発行日若しくは他の特別な理由を確立するために引用する文献（理由を付す）
「O」口頭による開示、使用、展示等に言及する文献
「P」国際出願日前で、かつ優先権の主張の基礎となる出願の日の後に公表された文献
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「X」特に関連のある文献であって、当該文献のみで発明の新規性又は進歩性がないと考えられるもの
「Y」特に関連のある文献であって、当該文献と他の1以上の文献との、当業者にとって自明である組合せによって進歩性がないと考えられるもの
「&」同一パテントファミリー文献

国際調査を完了した日	国際調査報告の発送日
04.07.01	17.07.01

国際調査機関の名称及びあて先	特許庁審査官（権限のある職員）	
日本国特許庁（ISA/JP） 郵便番号100-8915 東京都千代田区霞が関三丁目4番3号	森井 隆信	4C 9455
	電話番号 03-3581-1101 内線 3451	

様式PCT/ISA/210（第2ページ）（1998年7月）

C（続き）. 関連すると認められる文献

引用文献のカテゴリー*	引用文献名 及び一部の箇所が関連するときは、その関連する箇所の表示	関連する請求の範囲の番号
Y	KLIEWER, Steven A., et al. Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc. Natl. Acad. Sci. U. S. A., 1997, Vol.94, No.9, pages 4318 to 4323 & Database CAPLUS on STN, AMERICAN CHEMICAL SOCIETY (ACS), (Columbus, OH, USA), DN.127:30658	1-13
Y	GOETTLICHER, Martin, et al. Structural and metabolic requirements for activators of the peroxisome proliferator-activated receptor. Biochem. Pharmacol., 1993, Vol.46, No.12, pages 2177 to 2184 & Database CAPLUS on STN, AMERICAN CHEMICAL SOCIETY (ACS), (Columbus, OH, USA), DN.120:159789	1-13
Y	ISSEMANN, I., et al. The peroxisome proliferator-activated receptor:retinoid X receptor heterodimer is activated by fatty acids and fibrate hypolipemic drugs. J. Mol. Endocrinol., 1993, Vol.11, No.1, pages 37 to47 & Database CAPLUS on STN, AMERICAN CHEMICAL SOCIETY (ACS), (Columbus, OH, USA), DN.119:263421	1-13
X Y	EP 194693 A1(EISAI CO. LTD.) 17.9月.1996(17.09.96) & US 4788330 A & US 4883916 A & JP 61-210050 A	8,9,12,13 8-13
Y	EP 54732 A1(EISAI CO. LTD.) 30.6月.1982(30.06.82) & US 4655973 A & JP 57-106638 A	8-13

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0270-7306/03/$08.00+0 DOI: 10.1128/MCB.23.23.8528–8541.2003

Positive and Negative Regulation of the Cardiovascular Transcription Factor KLF5 by p300 and the Oncogenic Regulator SET through Interaction and Acetylation on the DNA-Binding Domain

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Here we show a novel pathway of transcriptional regulation of a DNA-binding transcription factor by coupled interaction and modification (e.g., acetylation) through the DNA-binding domain (DBD). The oncogenic regulator SET was isolated by affinity purification of factors interacting with the DBD of the cardiovascular transcription factor KLF5. SET negatively regulated KLF5 DNA binding, transactivation, and cell-proliferative activities. Down-regulation of the negative regulator SET was seen in response to KLF5-mediated gene activation. The coactivator/acetylase p300, on the other hand, interacted with and acetylated KLF5 DBD, and activated its transcription. Interestingly, SET inhibited KLF5 acetylation, and a nonacetylated mutant of KLF5 showed reduced transcriptional activation and cell growth complementary to the actions of SET. These findings suggest a new pathway for regulation of a DNA-binding transcription factor on the DBD through interaction and coupled acetylation by two opposing regulatory factors of a coactivator/acetylase and a negative cofactor harboring activity to inhibit acetylation.

The Sp/KLF (for Sp1- and Krüppel-like factor) family of zinc finger transcription factors has received recent attention due to important roles in developmental, differentiation, and oncogenic processes, among others (2, 3, 35). It is comprised of over 15 mammalian family members which have in common three similar C_2H_2-type zinc fingers at the carboxyl terminus which comprises the DNA-binding domain (DBD). Sp/KLF family members include the founding ubiquitous factor Sp1 (9), the erythroid differentiation factor EKLF/KLF1 (27), and the tumor suppressor gene KLF6/GBF/Zf9/COPEB, which we and others identified as a cellular factor possibly involved in human immunodeficiency virus type 1 transcription (18, 32, 44). It was recently shown by gene knockout studies that the proto-oncogene KLF5/BTEB2/IKLF (40, 42) is important for cardiovascular remodeling in response to stress (41). Contrary to initial expectations that this family of factors would likely have redundant functions, they in fact have important individual biological functions. However, the underlying mechanisms governing their specific functions and regulation are poorly understood.

We have studied the regulatory mechanisms of action of Sp/KLF family members in the past and have shown differential regulation through interaction and acetylation on the DBD by the coactivator/acetylase p300 (45). Acetylation is an important nuclear regulatory signal which regulates transcriptional processes with biological implications, including regulation of development, differentiation, and oncogenesis (5, 10, 31), which closely resembles the roles of Sp/KLF family members. We therefore thought that the Sp/KLF factors may be differently regulated by acetylation and showed that the coactivator/acetylase p300, but not the MYST-type acetylase Tip60, specifically interacts and acetylates Sp1 but not KLF6 through the zinc finger DBD and that DNA binding inhibits this interaction and acetylation (45). While much is known of acetylation in general, its regulation and implications are still poorly understood, especially its negative regulation.

Studies on negative regulation of acetylation have been centered mainly on the role of histone deacetylases, which are categorized into three classes based on sequence characteristics, subcellular localization, and catalytic properties (17, 33). We have shown an additional pathway involving negative regulation by DNA binding (45), and others have shown that a set of molecules inhibit the acetylation of histones by masking the protein from acetylation (e.g., inhibitors of histone acetylation [INHAT]) (39). Acetylation is therefore regulated at multiple levels by both catalytic and noncatalytic processes.

Here we show a regulatory pathway of acetylation involving the oncogenic regulator SET, a subunit of a complex previously shown to inhibit histone acetylation by masking the protein (INHAT) (39), through interaction with the DNA-binding transcription factor KLF5. Our findings suggest a new tran-

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scriptional regulatory pathway through the DBD by convergence of two opposing regulatory pathways involving p300 and SET through coupled interaction and acetylation.

MATERIALS AND METHODS

Preparation of recombinant epitope-tagged protein. The zinc finger region/DBD of human KLF5 (KLF5 ZF/DBD) (40) (a kind gift of C. Teng) was PCR amplified and subcloned into *Bam*HI-digested 6His-pET11d (45). Protein expression and purification were done essentially as described previously, with modification by use of HiTrap heparin and HisTrap columns (Amersham Pharmacia Biotech) (45). Glutathione *S*-transferase (GST)-tagged constructs KLF5 wt (wild type), KLF5-ΔDBD, KLF5-DBD, KLF5-zinc finger 1, KLF5-zinc finger 2, and KLF-zinc finger 3 were similarly PCR amplified and inserted into pGEX vectors (Amersham Pharmacia Biotech) (44, 45). Hexahistidine-tagged SET/TAF-Iβ construct (29) (a kind gift of K. Nagata) was transformed into the BL21-Gold(DE3)pLysS strain, induced, and then purified with Probond resin (Invitrogen) and buffer C containing 20 mM imidazole for washing and 200 mM imidazole for elution similar that described previously (45). SET/TAF-Iβ deletion constructs were in part a generous gift from K. Nagata, otherwise they were constructed by PCR mutagenesis. The p300 histone acetyltransferase (HAT) domain constructs have been described previously (45) (a kind gift of Y. Nakatani). All procedures were done at 4°C. Zinc finger peptides were synthesized commercially by Hokkaido System Science Co. Ltd. Product purity was more than 95%, and the molecular weights of synthesized peptides were confirmed by mass spectral analysis.

Cell culture and preparation of nuclear extract. C2/2 rabbit vascular smooth muscle cells (VSMCs) (48) were maintained in Dulbecco's modified Eagle medium (Sigma), and HeLa S3 cells were maintained in Joklik's modified Eagle medium (Sigma) supplemented with 10% fetal bovine serum with 100 μg of streptomycin/ml and 100 U of penicillin G/ml. Nuclear extract was prepared as described previously (7). The final supernatant was dialyzed against buffer B (25 mM HEPES [pH 7.9], 10% glycerol, 150 mM KCl, 100 μM $ZnSO_4$, 5 mM 2-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, 0.5 μg of leupeptin/ml, and 1 μg of pepstatin/ml), centrifuged for 15 min at 18,000 × *g*, and used as nuclear extract.

Isolation of factors associating with KLF5 ZF/DBD. Fifty micrograms of hexahistidine-tagged KLF5 ZF/DBD was bound to 5 μl of equilibrated Probond nickel-chelating resin (Invitrogen) by rotating for 6 h. Following five washes with buffer B, the resin bound with KLF5 ZF/DBD was then incubated for 6 h with 725 μg of C2/2 nuclear extract. After 10 washes with buffer B containing 20 mM imidazole, the bound proteins were eluted with buffer B containing 500 mM imidazole. Samples were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Coomassie brilliant blue staining. All procedures were done at 4°C.

Protein identification by MALDI-TOF (MS). Protein bands were excised from a Coomassie brilliant blue-stained SDS-PAGE gel and washed three times in 50% acetonitrile–25 mM ammonium bicarbonate (pH 8), and 100% acetonitrile was added. Following removal of acetonitrile, the gel slices were dried and the protein band was in-gel digested with 15 μg of trypsin/ml in 25 mM ammonium bicarbonate (pH 8) at 37°C overnight. Gel slices were then soaked in 50% acetonitrile–5% tetrahydrofuran, and the supernatant was collected and dried up. The dried digest was reconstituted by the addition of 50% acetonitrile–0.1% tetrahydrofuran, mixed with α-cyano-4-hydroxycinnamic acid, and analyzed by matrix-assisted laser desorption–ionization time of flight (mass spectrometry) [MALDI-TOF (MS)] (Voyager-DE STR; Applied Biosystems). Database searches were performed against the nonredundant National Center for Biotechnology Information database by using Protein Prospector programs, version 3.2.1, developed by the University of California—San Francisco MS facility. MS analysis of peptide acetylation was done essentially as described previously (16).

Protein-protein interaction assay. GST fusion proteins were immobilized to glutathione-Sepharose 4B resin and incubated with histidine-tagged proteins in a buffer containing 20 mM HEPES (pH 7.6 at 4°C), 20% glycerol, 0.2 mM EDTA, 0.1% Triton X-100, 100 mM NaCl, and 100 μM $ZnSO_4$. Reactions were carried out at 4°C for 3 h, and the mixtures were washed two times in the same buffer. Bound proteins were resolved on an SDS-PAGE (10% polyacrylamide) gel, transferred to a nitrocellulose membrane, immunoblotted with anti-HIS probe (G-18) antibody (Santa Cruz Biotechnology) or anti-FLAG (M2) antibody (Sigma), and detected with enhanced chemiluminescence Western blotting detection reagents essentially according to the manufacturer's instructions (Amersham Pharmacia Biotech). Histidine tag pull-down assays were done with Probond resin (Invitrogen) and blotted with anti-GST antibody (Santa Cruz) when appropriate.

Coimmunoprecipitation assay. One microgram of anti-KLF5 rat monoclonal antibody (KM1785) (41) or 1 μg of control rat immunoglobulin G (IgG) (sc-2026; Santa Cruz Biotechnology) was bound to 10 μl of protein G-Sepharose (Amersham Pharmacia Biotech) by rotating for 6 h in buffer B at 4°C. Following three washes with buffer B, protein G-Sepharose with antibody was then rotated with 1 mg of cell extract protein for 6 h. After 10 washes with radioimmunoprecipitation assay buffer, washed immunoprecipitates were subjected to SDS-PAGE and immunoblotting with SET/TAF-Iβ-specific antibody (a generous gift of K. Nagata) (30).

Immunoconfocal fluorescence microscopy. Murine VSMCs were prepared from the thoracic aortas of 8-week-old mice according to the enzyme digestion method (4). Cells were plated on glass coverslips, fixed with 4% formaldehyde, blocked with 3% bovine serum albumin in phosphate-buffered saline–Tween 20, incubated with anti-SET/TAF-Iβ mouse monoclonal antibody (a kind gift of K. Nagata) (30) and anti-KLF5 rat monoclonal antibody (KM1785), exposed to anti-mouse IgG antibody conjugated with fluorescein isothiocyanate and anti-rat IgG antibody conjugated with rhodamine isothiocyanate as the secondary antibodies, and then examined by confocal microscopy with a Leica TCS 4D equipped with an argon-krypton laser.

Gel shift DNA-binding assay. The gel shift DNA-binding assay was done essentially as described previously (45). A DNA oligomer containing the KLF5 binding sequence, 5′-ATGGGCATGAGGGCCAGCCTATGAGA-3′ (SE1), was used to analyze the DNA binding of KLF5 ZF/DBD (48). For mutant analysis, the underlined nucleotides GGGCC were replaced by TTTAA. For control NF-κB gel shifts, commercially available NF-κB probe (Promega) and recombinant NF-κB p50 protein (Promega) were used. The details (e.g., protein combinations) of individual experiments are given in the figure legends.

Cotransfection reporter assay. Plasmid constructs for the reporters, SMemb/NMHC-B and PDGF-A chain, and the effector pCAG-KLF5 have been described previously (41, 48). pCHA-SET/TAF-Iβ was a kind gift of K. Nagata (30). pCI-p300 and pCI-p300ΔHAT constructs were kind gifts of V. Ogryzko. Transient transfection assays were done by seeding cells (50,000 cells/24-well plate) 24 h prior to transfection and then transfected with 100 ng of the reporter plasmid and a total of 1 μg of either vector, pCAG-KLF5, or pCHA-SET/TAF-Iβ in combination, as described in the figure legends, by liposome-mediated transfer (Tfx-20; Promega) according to the manufacturer's instructions. Cells were harvested after 48 h and then subjected to an assay of luciferase activity (luciferase assay system; Promega) (Lumat LB9501; Berthold). The luciferase activity was normalized to the protein concentration of the cell lysates measured according to the Bradford method (Bio-Rad). For NF-κB control experiments, commercially available NF-κβ reporter was used (Stratagene), with coexpression of NF-κβ p50 and p65 expression plasmids (K. Aizawa, T. Suzuki, N. Kada, A. Ishihara, K. Kowase, T. Matsumura, K. Sasaki, Y. Munemasa, I. Manabe, M. Kurabayashi, T. Collins, and R. Nagai, submitted for publication).

Construction of point mutant expression construct. Site-directed mutagenesis was used to construct the mutant pCAG/KLF5-K369R, which involves a lysine-to-arginine mutation at amino acid residue position 369 of KLF5. PCR was done with the primers 5′-GACGACCATCCACTACTGCGATT-3′ and 5′-TCTCCA AATCGGGGTTACTCCTT-3′, with pCAG/KLF5 as a template and KOD Plus (Toyobo) polymerase. The construct was sequenced for verification.

Construction of recombinant adenovirus vectors. KLF5, the KLF5 K369R mutant, and SET cDNA were subcloned into the adenovirus cosmid vector pAxCAwt (Takara) at the *Swa*I site. 293 cells were cotransfected by the cosmid vector and restriction enzyme-treated DNA-terminal protein complex, with subsequent selection of plaques as a result of homologous recombination. The protein expression was confirmed by Western blot analysis. The titer was determined by the plaque method.

Production of stable transformant cell lines. KLF5 and KLF5 K369R cDNA inserts were subcloned into 3× FLAG expression vectors (Sigma). Constructs were transfected into cells (3T3-3) and then selected on the basis of G418 resistance for 2 weeks.

BrdU incorporation assay. Cells were plated on 96-well plates. Following adenovirus-mediated transfection in given experiments, bromo-2′-deoxyuridine (BrdU) incorporation was examined after 24 h over a span of 2 h by using the Biotrak cell proliferation enzyme-linked immunosorbent assay system, version 2 (Amersham Pharmacia Biotech), essentially according to the manufacturer's instructions. Experiments were done in triplicate.

Phorbol ester-induced expression of KLF5 and SET. Cell lysates from C2/2 VSMCs stimulated with phorbol 12-myristate 13-acetate (PMA) (100 ng/ml) following 24 h of starvation (0% fetal bovine serum) were resolved on an SDS–12% PAGE gel, transferred to a nitrocellulose membrane, and then im-

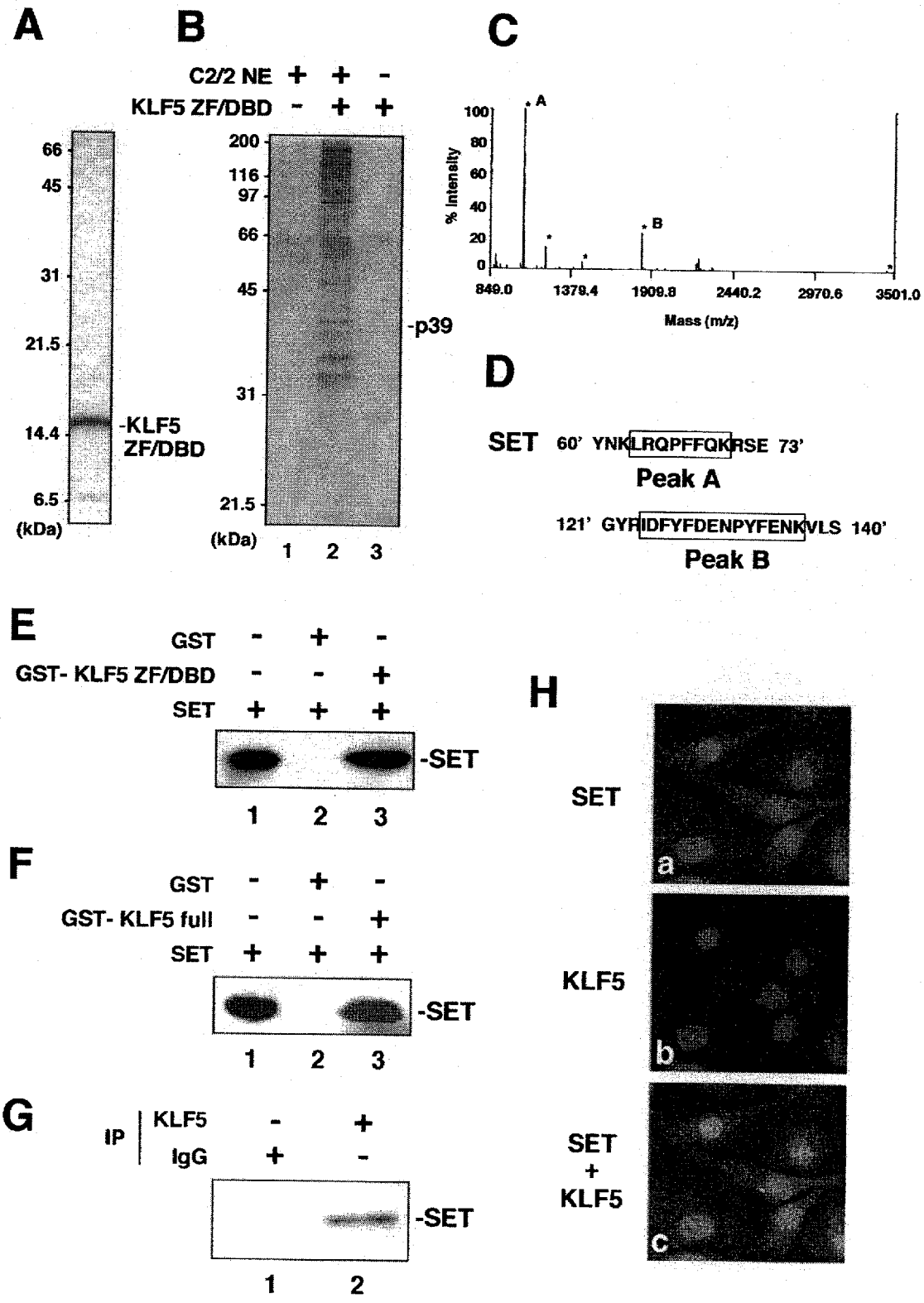


FIG. 1. Isolation of interactors of KLF5. (A) Silver-stained gel of the histidine-tagged KLF5 ZF/DBD recombinant used for interaction studies. Molecular mass markers are shown on left. (B) Isolation of factors associating with KLF5 ZF/DBD. Histidine-tagged KLF5 ZF/DBD was bound to nickel-chelating resin and subjected to VSMC C2/2 nuclear extract (NE) (lane 2). Lane 1 is nuclear extract alone, and lane 3 is recombinant protein immobilized on resin alone. Eluate was resolved by SDS-PAGE (12% polyacrylamide) and stained by Coomassie brilliant blue. The stained band, which was excised and subjected to further analysis, is indicated p39. (C) MALDI-TOF mass spectra obtained from tryptic peptides of p39.

munoblotted with anti-KLF5 monoclonal antibody (KM1785) or anti-SET/TAF-Iβ antibody (30). The relative intensity of KLF5 or SET protein in reference to Coomassie brilliant blue stain was calculated by using National Institutes of Health Image software. Total RNA was obtained by the RNeasy preparation kit (Qiagen) and reverse transcribed, and then quantitative PCR was performed with a platelet-derived growth factor A (PDGF-A) chain gene-specific primer set (5′-CAGCATCCGGGACCTCCAGCGACTC-3′ and 5′-TCGTAAATGACCG TCCTGGTCTTGC-3′) and a QuantumRNA 18S internal standard primer set (Ambion) as previously described (22). The relative intensity of the PDGF-A chain in reference to internal 18S rRNA was calculated by National Institutes of Health Image software.

Rat arterial injury model and immunohistochemistry. After 4 weeks on their respective diets, rats weighing 400 to 450 g were anesthetized with chloral hydrate (370 mg/kg of body weight, intraperitoneally). Balloon denudation of the left common carotid artery was performed. The right common carotid artery served as a control. Fourteen days after operation, rats were euthanized with a lethal dose of anesthetic, after which the carotid arteries were perfused with 4% paraformaldehyde and phosphate-buffered saline. Each injured left carotid artery was excised from the proximal edge of the omohyoid muscle to the carotid bifurcation. The middle third of the segment was then fixed in 4% paraformaldehyde for 12 h and embedded in paraffin. Serial cross sections (6 μm thick) were cut from each sample and stained with hematoxylin-eosin or prepared for immunohistochemistry. For immunohistochemical analysis, tissue sections were preincubated with 2% bovine serum albumin and then serially treated with SET-specific antibody (KM1720) (30) or KLF5-specific antibody (KM1785). Specimens were then treated with biotinylated goat anti-mouse IgG antibody (Vector Laboratories) or biotinylated goat anti-rat IgG antibody (Chemicon) followed by avidin-biotinylated horseradish peroxidase (Vectastain ABC kit; Vector Laboratories) and developed with 0.004% H_2O_2 and 0.02% diaminobenzidine tetrahydrochloride.

RESULTS

Isolation of interactors of KLF5. To isolate factors which regulate KLF5 by protein-protein interaction, we affinity purified interacting factors by using the ZF/DBD region, which is a potent protein-protein interface (21) that has been previously shown to mediate differential interaction with acetylase (45). Nuclear extract obtained from C2/2 VSMCs which express KLF5 were applied to hexahistidine-tagged recombinant KLF5 ZF/DBD immobilized on Ni^{2+}-chelating resin (Fig. 1A). Bound proteins were released by imidazole and then analyzed by SDS-PAGE with Coomassie brilliant blue staining (Fig. 1B). Approximately 20 bands ranging from 30 to 200 kDa were seen when the binding reaction between the KLF5 ZF/DBD and nuclear extract was done (Fig. 1B, lane 2) and not when either nuclear extract or KLF5 ZF/DBD alone (Fig. 1B, lanes 1 and 3) was used.

One band with an apparent molecular mass of 39 kDa was found in abundance and easily discernible from other nearby bands. MALDI-TOF (MS)–peptide mass fingerprinting, with a computer search of the National Center for Biotechnology Information mammalian database with further confirmation of the amino acid sequence by postsource decay peptide sequencing, revealed this protein to be the SET oncoprotein, the product of the SET oncogene whose translocation has been implicated in leukemia (Fig. 1C and D) (1, 47). SET is identical to the cellular factor template activating factor-Iβ which stimulates adenoviral core DNA replication (24, 29) and has been shown to be a histone chaperone, which is a factor that can displace and/or assemble nucleosomal histones in an ATP-independent manner (15, 25, 26, 34). While interaction of histone chaperones with histones and their activities to assemble and disassemble nucleosomal histones have been well addressed, their interaction with DNA-binding transcription factors has not been explored. Further, their cellular functions are poorly understood. Thus, characterization of this new interaction would add to our understanding of how histone chaperones may be involved in specific transcription by cooperative interaction with transcription factors as well as their cellular functions.

In vitro and in vivo interactions of KLF5 and SET. To determine whether SET directly interacts with KLF5 ZF/DBD, a GST pull-down assay was done (Fig. 1E). SET bound KLF5 ZF/DBD (Fig. 1E, lane 3) but not GST alone (Fig. 1E, lane 2), showing that SET and KLF5 ZF/DBD bind directly. To next see whether full-length KLF5 binds SET, a similar GST pull-down assay with full-length KLF5 was done (Fig. 1F), which showed that SET also binds full-length KLF5 (Fig. 1F, lane 3). To further see whether KLF5 and SET interact in the cell, immunoprecipitation was done with specific antibodies against KLF5 and SET (Fig. 1G). Immunoprecipitation of KLF5 followed by immunoblotting with SET antibody showed detection of SET when antibody against KLF5 was used (Fig. 1G, lane 2) but not for control IgG antibody (Fig. 1G, lane 1). SET therefore interacts with KLF5 in vitro and in vivo.

To further examine the expression and cellular localization of SET, immunohistochemistry was done (Fig. 1H). SET (Fig. a) and KLF5 (Fig. b) colocalized to the nucleus in an overlapping pattern (Fig. c), which is supportive of functional interaction.

Functional effects of interaction of KLF5 and SET. To next address the functional implications of the interaction of SET and KLF5, we first examined the effects on the DNA-binding activity of KLF5 (Fig. 2A). Gel shift analysis under conditions in which KLF5 ZF/DBD showed sequence-specific binding and SET did not bind the probe DNA (Fig. 2A, lanes 2 to 4) showed inhibition of KLF5 DNA-binding activity by the addition of SET (Fig. 2A, lanes 7 and 8). SET did not inhibit the DNA-binding activity of the control NF-κB p50 subunit (Fig. 2B).

To further examine the effects of SET on KLF5-dependent

Fragment peaks assigned to SET are marked (asterisks). Peaks indicated A and B were subjected to postsource decay sequencing. (D) Partial peptide sequences of human SET. Numbering is from the initiation methionine of SET. The peptide sequences of peaks A and B obtained by postsource decay are boxed. (E) In vitro binding of KLF5 ZF/DBD and SET. Immobilized GST-KLF5 ZF/DBD fusion protein was reacted with histidine-tagged SET protein, separated by SDS-PAGE, and analyzed by immunoblotting with anti-HIS probe antibody (lane 3). Lane 1 is the input. GST protein was used as the control (lane 2). (F) In vitro binding of KLF5 full-length protein and SET. Immobilized GST-KLF5 full-length fusion protein was reacted with histidine-tagged SET protein, separated by SDS-PAGE, and analyzed by immunoblotting with anti-HIS probe antibody (lane 3). Lane 1 is the input. GST protein was used as a control (lane 2). (G) Coimmunoprecipitation of SET with KLF5. Cell lysate was immunoprecipitated with anti-KLF5 rat monoclonal antibody (lane 2) or normal rat IgG (lane 1) as a control. Bound materials were separated by SDS-PAGE and analyzed by immunoblotting with anti-SET antibody. (H) Intracellular localization of KLF5 and SET. Endogenous SET (a) (green) and KLF5 (b) (red) were detected mainly in nuclei. Confocal microscopy double-staining analysis indicates colocalization of SET and KLF5 (c) (yellow). All experiments were done at least twice with consistent findings. +, present; −, absent.

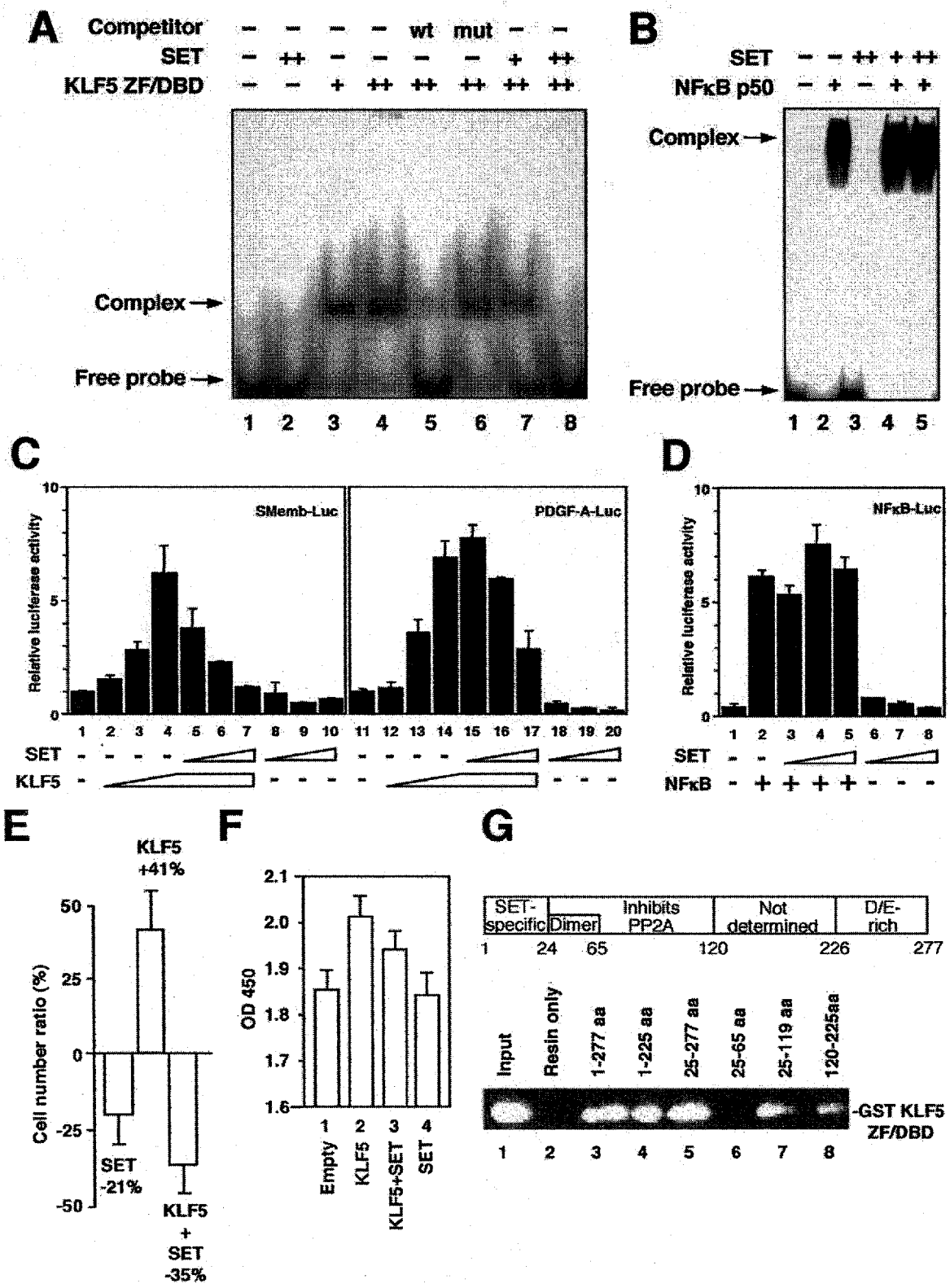


FIG. 2. Effects of SET on KLF5 activity. (A) Effects of SET on KLF5 DNA-binding activity. A gel shift assay with recombinant KLF5 ZF/DBD and SET was performed. Wt and mut represent wild and mutant oligonucleotide competitors (lanes 5 and 6). The amount of recombinant protein is as follows: 150 (+) and 450 (++) ng for SET (lanes 2, 7, and 8) and 10 (+) and 50 (++) ng for KLF5 ZF/DBD (lanes 3 to 8). (B) Gel shift assay of control NF-κB p50 subunit and SET. Gel shift units (0.1) of NF-κB p50 (lanes 2, 4, and 5) and 100 (+) and 300 (++) ng of SET (lanes 3, 4, and 5) were used. (C) Effects of SET on KLF5 transactivation. Cotransfection analysis of effects of SET on KLF5 transcriptional activation.

transcriptional activation, cotransfection reporter assays were done (Fig. 2C). Using the originally identified KLF5-responsive embryonic vascular smooth muscle (SMemb) promoter (48) and the PDGF-A chain promoter, which is an endogenous target of KLF5 (41), under conditions in which KLF5 showed dose-dependent transactivation of the SMemb and PDGF-A chain promoter activities (Fig. 2C, lanes 2 to 4 and 12 to 14) and SET did not show activation of the respective promoters (Fig. 2C, lanes 8 to 10 and 18 to 20), cotransfection of SET showed dose-dependent inhibition of KLF5-mediated transactivation (Fig. 2C, lanes 5 to 7 and 15 to 17). SET did not inhibit transactivation by NF-κB (Fig. 2D). SET therefore negatively regulates both KLF5 DNA-binding and transactivation activities in a specific manner.

The effects of SET on KLF5 cellular activity were further addressed given that KLF5 is a proto-oncogene which accelerates cell growth (Fig. 2E) (43). Under conditions in which adenovirus-mediated forced expression of KLF5 stimulated the growth of C2/2 cells, expression of SET inhibited cell growth in KLF5-expressing cells to almost basal levels, suggesting coordinated effects of SET and KLF5 on cell growth. To note, SET alone also showed inhibitory effects on cell growth. A BrdU assay further confirmed that SET inhibited cell growth as induced by KLF5 (Fig. 2F).

To determine the region of SET which interacts with KLF5, deletion mutants were made and subjected to a pull-down assay (Fig. 2G). Results collectively showed that a 40-amino-acid stretch (amino acids 25 to 65) known as a coiled-coil dimerization domain of SET (28) did not interact with KLF5, but otherwise, a broad region of SET interacted with KLF5. Initial results with a SET mutant which does not interact with KLF5 did not show effects on cell growth, suggesting that interaction is important for cooperative effects of SET and KLF5 on cell growth (data not shown).

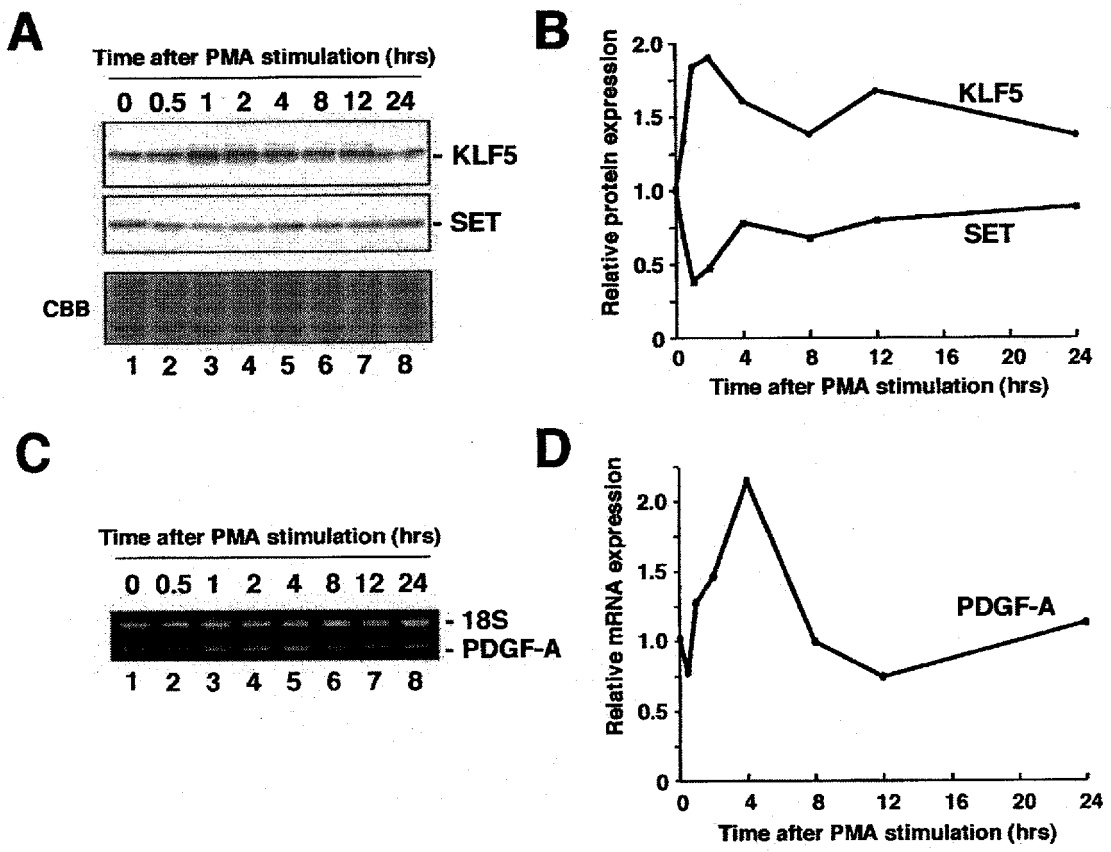
Biological implications of interaction of KLF5 and SET. To further examine the biological implications of the interaction of KLF5 and SET, we assessed the effects of SET on KLF5-dependent gene expression. KLF5 and its downstream gene PDGF-A chain are induced by various stimuli (e.g., phorbol ester, angiotensin II, and serum) (14, 48). We examined whether SET expression could also be similarly regulated. Expression levels of KLF5, SET, and PDGF-A chain were assessed at various time intervals after PMA stimulation by Western blotting for KLF5 and SET, and reverse transcription-PCR analysis for the PDGF-A chain (Fig. 3A to D). An induction of KLF5 protein was seen at 2 h with a 1.9-fold increase after PMA stimulation, with a coinciding 2.5-fold decrease in SET protein levels (Fig. 3A and B). PDGF-A mRNA levels showed a 2.2-fold increase at 4 h (Fig. 3C and D). KLF5, SET, and PDGF-A chain levels returned to basal levels at 24 h. Collectively, reciprocal increased KLF5 and coinciding decreased SET correlated with increased PDGF-A chain expression after PMA stimulation.

We further examined expression of KLF5 and SET in pathological states by histopathological analysis. An experimental atherosclerosis model (balloon injury) was used in which KLF5 is activated in proliferating neointimal smooth muscle cells after injury (11, 41). Balloon-injured aortas and controls at 2 weeks were examined for expression of KLF5 and SET by immunohistochemistry (Fig. 3E). In contrast to low basal levels of either SET or KLF5 in the nucleus of noninjured control aortic medial cells, proliferating neointimal cells, which form after injury and consist mainly of proliferating smooth muscle cells, showed marked expression of both KLF5 and SET in the nucleus. These findings suggest correlation and colocalization of KLF5 and SET in pathological states and support a functional interaction at the tissue or animal level.

Inhibition of KLF5 acetylation by SET. SET regulates the actions of KLF5 as determined from these studies, but the underlying mechanisms were still not fully understood. One of the functions of SET has been recently shown to be inhibition of histone acetylation (39). It has been shown in the past that the KLF5 family member Sp1 is acetylated by p300 in the ZF/DBD region (45), and as Sp1 and KLF5 have similar ZF/DBDs, and further because SET interacted with the ZF/DBD of KLF5, we reasoned that KLF5 may also be similarly acetylated, and if so, be inhibited by SET. By such, SET may negatively regulate the actions of KLF5 by blocking acetylation.

First, we examined whether KLF5 can be acetylated by pulse-chase experiments (Fig. 4A). Using [^{3}H]acetate and the histone deacetylase inhibitor trichostatin A, cells expressing KLF5 showed a clear uptake, thus showing that KLF5 can be acetylated in vivo. We next examined which region of KLF5 is acetylated by an in vitro acetylation assay by using acetyl [^{14}C]coenzyme A (acetyl-[^{14}C]CoA) (Fig. 4B). Using a catalytic recombinant protein of p300 which acetylates the similar factor Sp1, full-length KLF5 and the KLF5 ZF/DBD region were acetylated but the non-ZF/DBD region was not, showing

One hundred nanograms of reporter was used in each lane. Effectors are as follows: lanes 2, 3, and 4 through 7, and 12, 13, and 14 through 17 were 83, 250, and 750 ng of KLF5 expression plasmid (pCAG-KLF5), respectively; lanes 5 and 8, 6 and 9, 7 and 10, 15 and 18, 16 and 19, and 17 and 20 were 28, 83, and 250 ng of SET expression plasmid (pCHA-SET/TAF-Iβ). The total amount of effector plasmid was adjusted to 1 μg with the respective control vector. (D) Effects of SET on control NF-κB transactivation. NF-κB transactivation (lanes 2 to 5) was done by transfection of equal amounts (250 ng) of p50 and p65 subunit expression vectors. (E) Effects of SET on KLF5-induced cell growth. SET was transiently transfected into cells stably expressing epitope-tagged (3× FLAG) KLF5 or mock vector in 3T3-3 cells. The cell count on day 5 after transfection compared with mock vector-treated cells is shown. Error bars denote standard errors. (F) BrdU assay showing effects of KLF5 and SET on cell growth by use of adenovirus-mediated transfer (multiplicity of infection, 100) of KLF5 and SET adenoviruses. Empty (lane 1) denotes empty vector alone. Error bars denote standard errors. OD_{450}, optical density at 450 nm. (G) In vitro binding of KLF5 ZF/DBD and SET deletion mutants. Immobilized histidine-tagged SET protein was reacted with GST-KLF5 ZF/DBD fusion protein, separated by SDS-PAGE, and analyzed by immunoblotting with anti-GST antibody. SET deletion mutants are shown by their amino acid numbers in reference to the schematic diagram of functional domains shown above. Lane 1 is GST KLF5 ZF/DBD input, and lane 2 shows that GST KLF5 ZF/DBD does not bind Probond nickel-chelating resin. Lanes 3 to 8 show GST KLF5 ZF/DBD binding to respective resin-bound deletion mutants. Amino acids (aa) 1 to 24 comprise the SET-specific N-terminal region, amino acids 25 to 65 comprise the coiled-coil dimerization domain, amino acids 25 to 119 comprise a region known to inhibit phosphatase PP2A, amino acids 120 to 225 comprise a region with unknown function, and amino acids 226 to 277 comprise the acidic C-terminal region. All experiments were done at least twice with consistent findings.



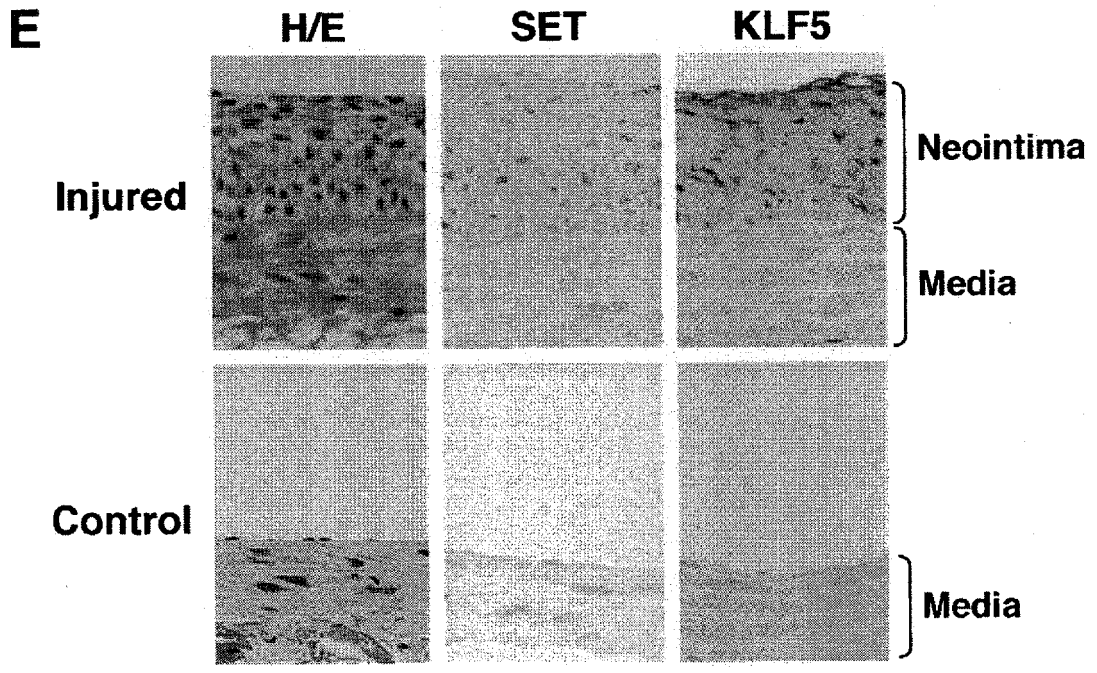


FIG. 3. Effects of SET on KLF5 downstream gene expression and pathological states. (A) Induction of KLF5 protein and repression of SET protein after mitogenic stimulation. Cells were starved at the times shown for 24 h, incubated with 100 ng of PMA/ml for the indicated times, and then harvested. Cell lysate was resolved by SDS-PAGE and subjected to Western blotting or Coomassie brilliant blue staining. (B) Quantification of KLF5 and SET protein levels. KLF5 and SET protein levels were normalized by the corresponding Coomassie brilliant blue staining pattern. The relative expression level was shown as the level at 0 h. (C) Induction of PDGF-A chain mRNA expression. Cells were starved at the times shown for 24 h, incubated with 100 ng of PMA/ml for the indicated times, and then harvested. The quantitative reverse transcription-PCR fragment

that the ZF/DBD region is acetylated similar to Sp1 (Fig. 4C). This was specific, as neither acetylase Tip60 (MYST domain containing Tat-interacting protein) nor GCN5 (homologue of mammalian PCAF) acetylated KLF5 ZF/DBD (data not shown).

To see whether acetylation affects the DNA-binding activity of KLF5, a gel shift assay was done under acetylation conditions which showed that acetylation of KLF5 has no effect on its DNA-binding activity (Fig. 4D). As p300 acetylated KLF5, we next examined whether SET could inhibit the acetylation of KLF5 (Fig. 4E). As expected, SET inhibited the acetylation of KLF5 ZF/DBD by p300. Order of addition experiments showed that SET was able to inhibit acetylation when reacted with KLF5 prior to the addition of acetylase, but it was unable to react if KLF5 and p300 were reacted beforehand, suggesting that SET inhibits acetylation of KLF5 by masking the protein or inducing a conformational change which does not allow for subsequent acetylation.

p300 as a transcriptional cofactor of KLF5. As the coactivator/acetylase p300 acetylated KLF5, we examined whether p300 acts as a coactivator of KLF5, that is, if it interacts with KLF5 and is able to potentiate KLF5-mediated transcriptional activation. First, to examine the interaction between KLF5 and p300, by use of immunoprecipitation with specific antibodies against KLF5 and p300, we show that, under conditions in which p300 is pulled down, immunoblot against KLF5 shows interaction of KLF5 with p300 (Fig. 5A). To next examine whether interaction is direct and by which region interaction is mediated, GST pull-down assays were done with KLF5 deletion mutants and the p300 acetylase catalytic region (Fig. 5B). Results showed that KLF5 ZF/DBD and full-length KLF5, but not non-ZF/DBD region KLF5, interact with p300. Thus, importantly, the ZF/DBD is the interacting domain with p300 in addition to the substrate for acetylation.

To further see whether p300 potentiates transactivation of KLF5, a cotransfection reporter assay was done (Fig. 5C). Under conditions in which KLF5 activated the PDGF-A chain reporter, the addition of p300 resulted in a dose-dependent increase in transactivation but transactivation did not occur with the addition of p300 alone, thus showing that p300 coactivates KLF5 transcription. p300 is therefore a coactivator of KLF5. A mutant of p300 with the acetylase catalytic region deleted showed reduced activation, suggesting that acetylation and/or interaction through this region with KLF5 is important for transactivation of PDGF-A chain reporter activity by KLF5.

Mapping of the acetylation site of KLF5. As SET inhibited acetylation of KLF5, we reasoned that understanding the effects of KLF5 acetylation would lead to a better understanding of the actions of SET. For this, we determined the acetylated residue(s) in KLF5 and used nonacetylated point mutants to understand the implications of acetylation in vivo. To determine the acetylation site within the KLF5 ZF/DBD, peptides of each of the three zinc fingers were prepared and subjected to acetylation reactions (Fig. 6A). The first zinc finger was the only peptide acetylated by p300 for both GST proteins and synthetic peptides (Fig. 6B and data not shown). MS of the peptides further showed that the first zinc finger peptide is monoacetylated, as shown by a single shifted peak of 42 *m/z* (Fig. 6C). The second and third zinc finger peptides did not show a shifted peak and were thus not acetylated in vitro (data not shown).

To next identify the acetylated lysine residue, the acetylated first zinc finger peptide was digested with Lys-C endopeptidase, which cleaves after nonacetylated but not acetylated lysine residues. Analysis of expected fragment masses against actual masses showed that the lysine residue at amino acid number 369 was acetylated (Fig. 6D). To further confirm that this is the only acetylated residue, this lysine was mutagenized to an arginine residue (hereafter referred to as a K369R substitution) to preserve the similar basic charge and then the peptide was subjected to an acetylation assay which showed that the arginine substitution resulted in loss of acetylation (Fig. 6E). SET can bind both K369R and wild-type KLF5, suggesting that interaction is not impaired by this mutation (Fig. 6F).

Functional implications of KLF5 acetylation. To examine the effects of acetylation on KLF5 function, the transcriptional activity and cellular effects of the K369R mutation were tested. Transcriptional activation examined by cotransfection reporter analysis showed that the K369R mutation showed no effects on PDGF-A chain promoter activity compared to wild-type KLF5 when transfected alone (Fig. 7A). SET also inhibited transactivation by KLF5 similarly for the wild type and the K369R mutant (Fig. 7B), suggesting that interaction is important for the inhibitory effects of SET on KLF5 transactivation. Masking acetylation is a likely result of this interaction. Further, a 20% decrease in PDGF-A chain promoter activity was seen when p300 was cotransfected with the K369R mutant KLF5 construct compared to the KLF5 wild-type construct (Fig. 7C), showing that acetylation is important for transactivation of PDGF-A chain reporter activity by p300 on KLF5.

To further examine the effects of acetylation on KLF5 cellular activity, effects on cell growth were investigated. In cells transfected with the wild type or the K369R mutant by adenovirus-mediated transfer, the K369R mutant-transfected cells showed a reduction in cell number (Fig. 7D). A BrdU assay also showed reduced uptake in the K369R mutant-transfected cells, further showing that cell replication is decreased in these cells (Fig. 7E). Interestingly, results of the effects of the nonacetylated K369R mutation of KLF5 closely mimicked the effects of SET on KLF5, suggesting

was resolved on a 2% agarose gel. (D) Quantification of mRNA expression level for the PDGF-A chain. The expression level of the PDGF-A chain, an endogenous target gene of KLF5, was normalized to that of 18S. (E) KLF5 and SET expression in the pathological neointima. The immunohistochemistry of SET and KLF5 in a balloon injury model of atherosclerosis was examined. The left common carotid artery was denudated by balloon injury, and the neointima was observed 2 weeks after the balloon injury (Injured). The right common carotid artery served as a control (Control). The rat aorta was stained with anti-SET and anti-KLF5 antibodies. Cells in the neointima were clearly positive for SET and KLF5. All experiments were done at least twice with consistent findings. H/E, hematoxylin and eosin staining.

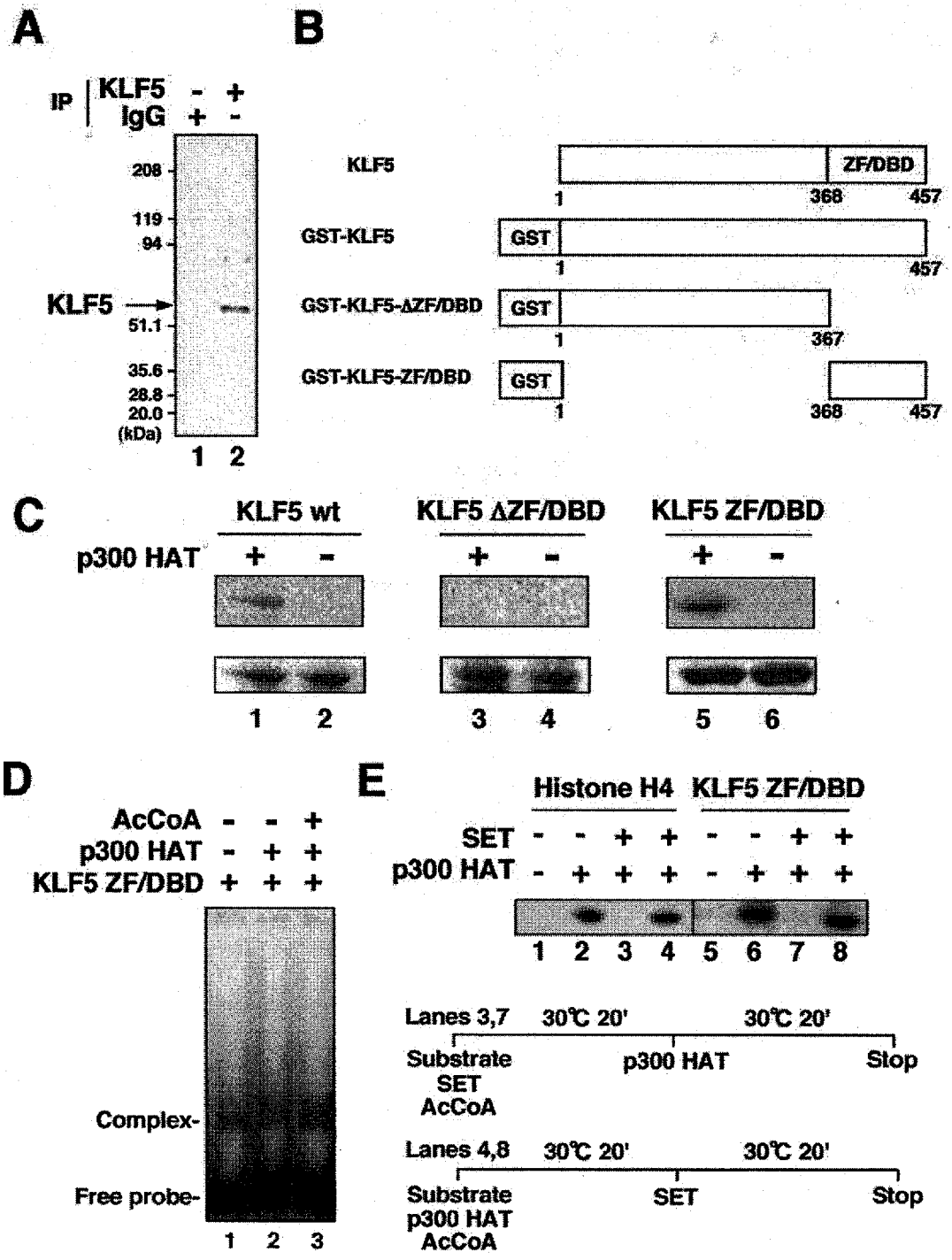


FIG. 4. Acetylation of KLF5 and its regulation by SET. (A) Acetylation of KLF5 in vivo. Cells were treated with trichostatin A and labeled with [^{3}H]acetate followed by immunoprecipitation with KLF5 (lane 2) and control normal IgG antibodies (lane 1). (B) Schematic representation of GST-KLF5 fusion mutant constructs. GST-KLF5 comprises full-length KLF5 fused to GST, GST–KLF5-ΔZF/DBD comprises only the N-terminal regulatory domain fused to GST, and GST–KLF5-ZF/DBD comprises only the C-terminal zinc finger DBD fused to GST. (C) Acetylation of KLF5 mutant constructs in vitro by p300. KLF5 proteins (1.2 μg) were incubated with 50 ng of FLAG-p300 HAT domain protein (amino acids 1195 to 1673) in the presence of [^{14}C]acetyl-CoA. Reaction products were separated by SDS–12% PAGE. The difference between pairs (lanes 1 and 2, 3 and 4, and 5 and 6) is the presence of p300 HAT protein in the reaction mixture for the respective KLF5 mutant proteins. The gel was stained with Coomassie brilliant blue (lower panel) and then analyzed with a BAS 1500 phosphorimager (upper panel). (D) Effects of acetylation on KLF5 DNA-binding activity. Acetylation reactions were performed in the presence (+) of acetyl-CoA (AcCoA) and FLAG-p300 HAT domain (lane 3), in the presence of FLAG-p300 HAT domain (lane 2), and in the absence (−) of acetyl-CoA or FLAG-p300 HAT domain (lane 1). Reaction products were resolved by electrophoresis and analyzed with BAS1500. (E) Effects of SET on KLF5 acetylation (lanes 5 to 8). Histone H4 was used as a control (lanes 1 to 4). A schematic diagram of the protocol for order-of-addition experiments is shown. In lanes 3 and 7, the p300 HAT domain was added following the reaction of SET with the substrate (KLF5 ZF/DBD or histone H4) (prior to acetylation), and in lanes 4 and 8, SET was added following the reaction of p300 HAT with the substrate (after acetylation). Acetylation reactions were done essentially as described above. All experiments were done at least twice with consistent findings.

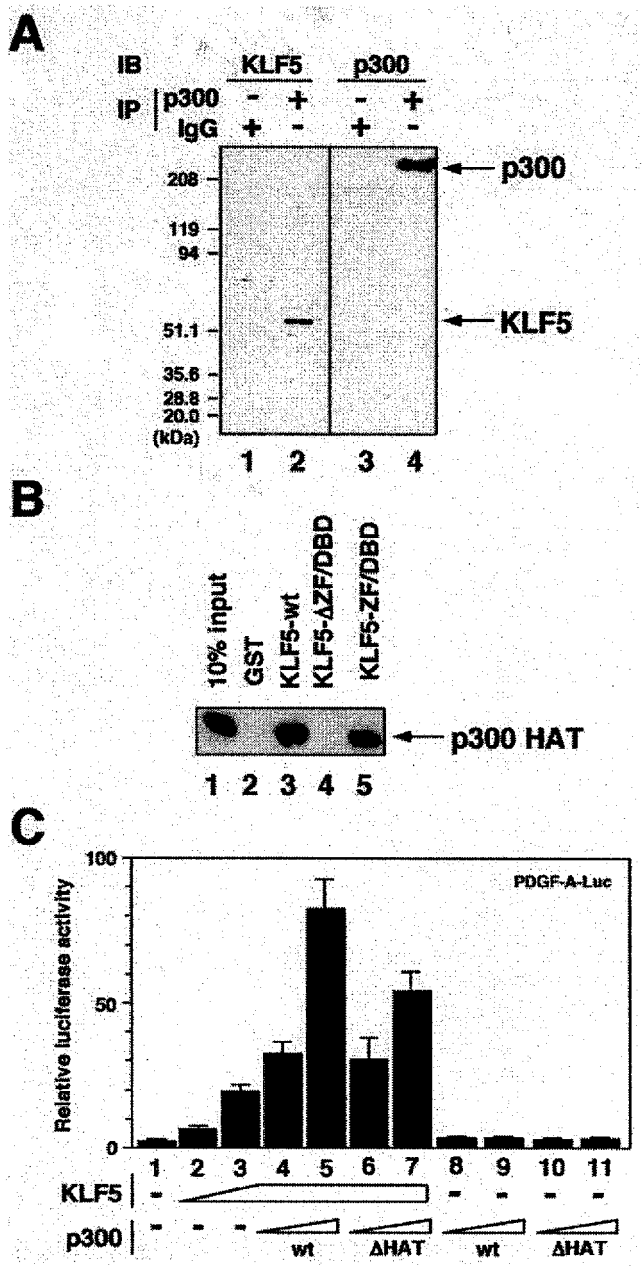


FIG. 5. Interaction and activation of KLF5 by the coactivator and acetylase p300. (A) Interaction of KLF5 and p300 in vivo. p300 was immunoprecipitated from cells followed by immunoblotting against KLF5 (lane 2). Immunoblotting against p300 confirms the immunoprecipitation procedure (lane 4). Normal IgG was used as the control (lane 2). (B) Interaction between KLF5 deletion mutants and p300 HAT domain in vitro. Approximately 2 μg of recombinant GST-KLF5 wild type (wt) (amino acids 1 to 457, lane 3), GST–KLF5-ΔDBD (amino acids 1 to 367, lane 4), and GST–KLF5-DBD (amino acids 368 to 457, lane 5) were used in a GST pull-down assay with 1 μg of the FLAG-p300 HAT domain. The interaction between KLF5 and the p300 HAT domain was analyzed by SDS–10% PAGE of the pull-down reactions and Western blotting with anti-FLAG antibody. The input (lane 1) contains 10% of the FLAG-p300 HAT domain protein. GST protein (lane 2) was used as a control. (C) Effects of p300 on KLF5 transactivation as assessed by reporter cotransfection assay. Cells were transfected with 100 ng of PDGF-A chain luciferase reporter and increasing amounts of KLF5 expression vector plasmids up to 750 ng (lanes 2 to 7). DNA concentrations were maintained constant by addition of the empty vector. Increasing amounts of p300 expression vector plasmids were similarly cotransfected up to 250 ng with 100 ng of PDGF-A–luciferase reporter plasmid in the absence (−) (lanes 8 and 9) or presence of 750 ng of KLF5 expression vector (lanes 4 and 5). Effects of an acetyltransferase region-deleted mutant of p300 (ΔHAT) on KLF5-mediated transcriptional activation were also examined (lanes 6, 7, 10, and 11). All experiments were done at least twice with consistent findings. +, present.

that the effects of SET on KLF5 may at least in part be by inhibition of KLF5 acetylation.

DISCUSSION

Regulatory pathway of coupled interaction and acetylation. We have described a new regulatory pathway of transcriptional activation and inhibition of a DNA-binding transcription factor through the DBD by coupled interaction and modification (e.g., acetylation) as shown through the opposing actions of p300 and SET on the transcription factor KLF5 (Fig. 7F). SET negatively regulates and p300 positively regulates KLF5 actions, and SET further inhibits acetylation of KLF5 by p300. Importantly, our biochemical and cellular data suggest that the effects of KLF5 acetylation are complementary to the effects of SET on KLF5 on the basis of regulation of transcriptional activation and cell growth. Our findings suggest that the actions of SET are therefore likely mediated at least in part by blocking activation as mediated through this chemical modification. The actions of SET on DNA-binding factors have been hitherto unknown, although past studies had addressed its role in adenoviral DNA replication and as a chaperone for histones (15, 25, 26, 34). The present findings therefore implicate a new role for SET in factor-specific regulation of transcription through a mechanism that has previously only been known for histones. As SET has been shown to similarly inhibit transcription of retinoic acid receptor transcription (39), it is tempting to speculate that this action can be generalized to include at least the nuclear receptor family, which contains a zinc finger-type DBD common to KLF5.

The regulation of acetylation is emerging as a new pathway for the control of transcriptional regulation. Catalytic regulation through actions of deacetylases (17, 33) and coupled regulation with other signaling pathways (i.e., regulation of p53 by coupled acetylation and phosphorylation) (38) and by other interactions has received much attention, but noncatalytic regulation by protein interaction with the substrate and/or enzyme is also a notable regulatory mechanism. SET, as a subunit of the INHAT complex (39), likely masks the substrate protein from acetylation by interaction (e.g., competition) and/or further inducing a conformational change, making it inaccessible to the enzyme. It was also previously shown that DNA binding inhibits interaction and acetylation of Sp1 by the acetylase p300 (45). Further, the human immunodeficiency virus-related tat protein has been shown to modulate acetylation, likely by interaction and by conformational changes to the substrate and/or enzyme, thus modulating acetylation activity and its effects on gene expression (6).

Taken together, modulation of acetylation through noncatalytic actions (e.g., blocking or masking interaction) is a pathway which can confer a new regulatory step to transcriptional regula-

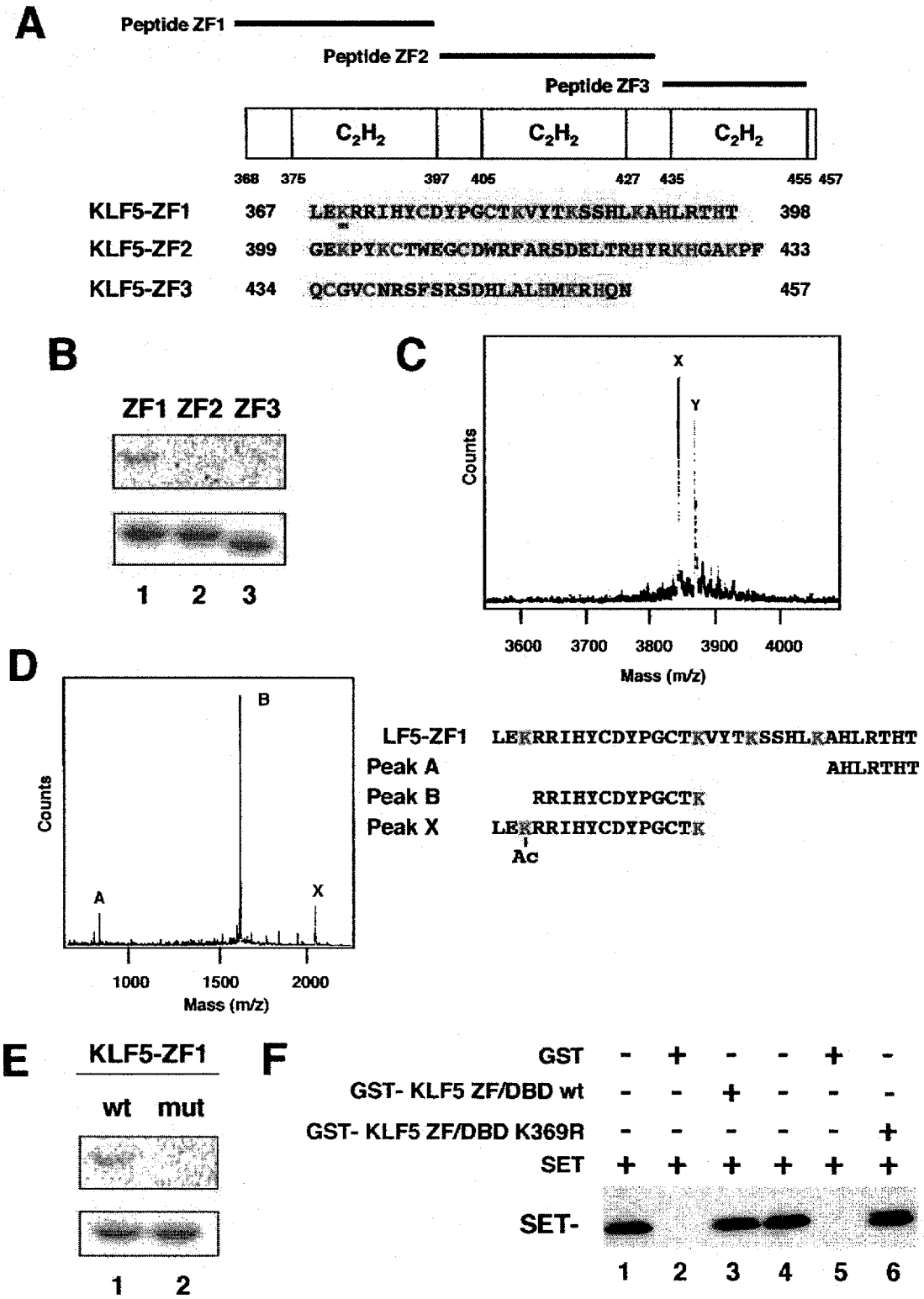


FIG. 6. Mapping of the acetylated region and residue of KLF5. (A) Schematic representation of KLF5 zinc finger peptides. ZF1, ZF2, and ZF3 cover each of the zinc fingers, respectively, from the N terminus. (B) Acetylation of KLF5 zinc finger mutants in vitro. Approximately 1.0 μg of purified GST-KLF5 fusion zinc fingers 1, 2, and 3 were incubated with [^{14}C]acetyl-CoA and recombinant FLAG-p300 HAT domain. Reaction products were separated by SDS–10% PAGE. The gel was stained with Coomassie brilliant blue (lower panel) and then analyzed with a BAS 1500 phosphorimager (upper panel). (C) Mass spectrum quantification of acetylated lysines in peptide KLF5-ZF1. A parallel reaction mixture with unlabeled acetyl-CoA was analyzed by MALDI-TOF (MS). The major peak labeled X corresponds to the expected mass of the unmodified peptide KLF5-ZF1. The major peak labeled Y, larger by 42 atomic mass units, represents monoacetylated peptide. (D) Masses of peptides digested with

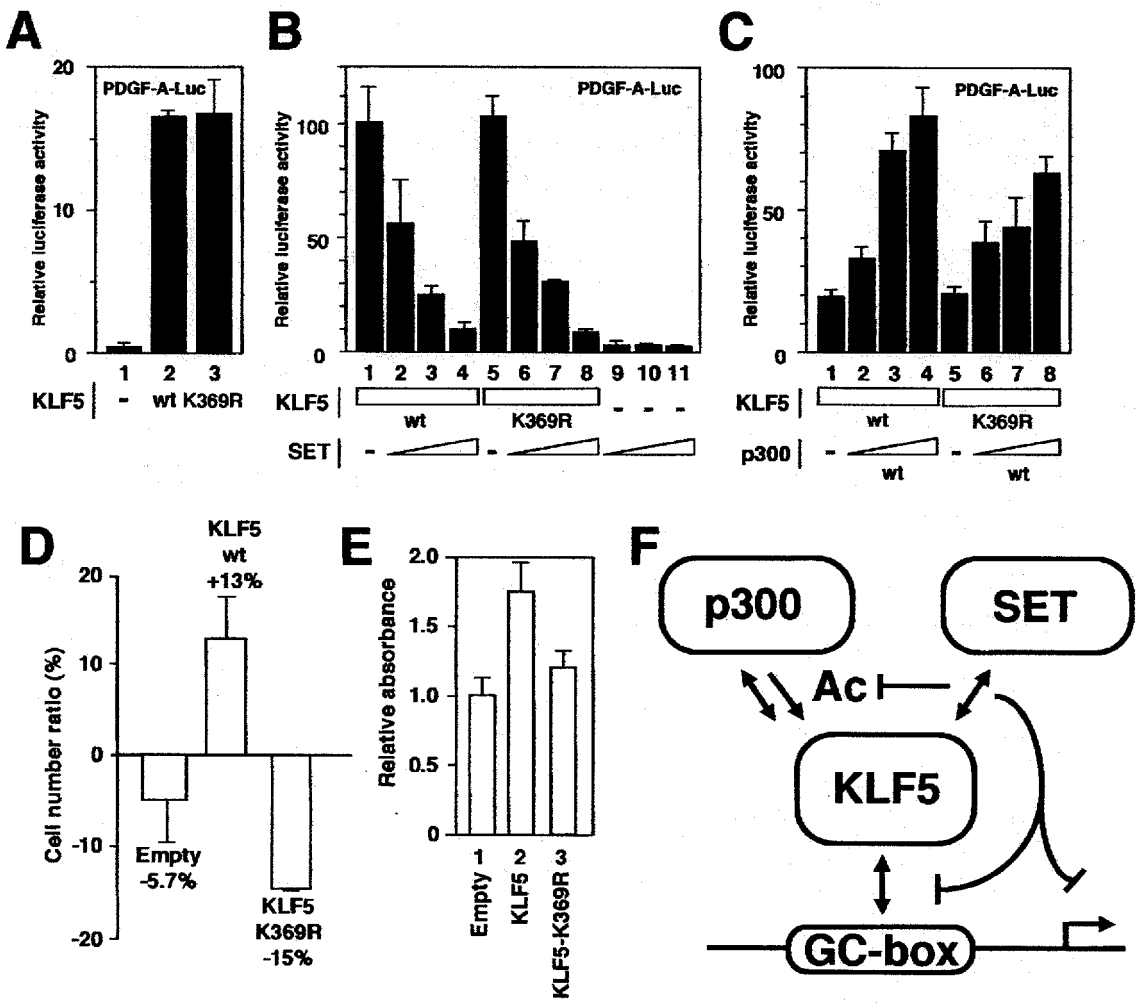


FIG. 7. Effects of the KLF5 K369R point mutant. (A) Effects of KLF5 K369R mutant (lane 3) on PDGF-A chain promoter transcriptional activation compared to that of the KLF5 wild type (wt) (lane 2). Seven hundred fifty nanograms of each expression vector was transfected in the presence of 100 ng of the reporter construct (all lanes). −, absent. (B) Effects of SET on KLF5 wild type and K369R mutant. Up to 250 ng of SET expression vector was transfected in the presence of 750 ng of KLF5 expression vector. (C) Effects of the KLF5 K369R mutant on PDGF-A chain promoter transcriptional activation compared to that of the KLF5 wild type in the presence of p300. Increasing amounts of p300 expression vector up to 250 ng were transfected in the presence of 750 ng of KLF5 (wild type, lanes 1 to 4; K369R mutant, lanes 5 to 8) expression vector and 100 ng of the reporter construct. (D) Effects of KLF5 K369R mutant on cell growth. The KLF5 wild type and K369R mutant were transfected with adenovirus and counted on the sixth day in comparison with nontreated cells. Error bars denote standard errors. (E) Effects of the KLF5 K369R mutant on cell growth were similarly assessed by BrdU assay. Error bars denote standard errors. All experiments were done at least twice with consistent findings. (F) Summary of findings. Note that SET negatively regulates DNA binding, transactivation, and acetylation of KLF5. p300 interacts, transactivates, and acetylates KLF5. We envision that this mechanism of interplay between coupled positive regulation by p300 and negative regulation by SET occurs in an inducible setting (e.g., phorbol ester stimulation).

tion of gene expression. Note, the combined role of noncatalytic acetylation blocking or masking events with those of deacetylases, which act following acetylation, and further regulation by other interacting regulatory proteins (e.g., complex) is still unknown. Future studies aimed at deciphering the complexity of the regulation of the collective transcription reaction as mediated by acetylation will be necessary to understand the precise role of this regulatory pathway.

Lys-C endopeptidase. The peptide sequences that are suggested from measured masses are shown below. Peak X represents the acetylated fragment. (E) Replacement of acetylated lysine by arginine impairs acetylation of GST–KLF5-zinc finger 1. Approximately 1.0 μg of purified GST–KLF5-zinc finger 1 (lane 1, wild type [wt]) and GST–KLF5-mut zinc finger 1 (K369R) (lane 2, mutant [mut]) were incubated with [^{14}C]acetyl-CoA and 50 ng of FLAG-p300 HAT domain protein. Reaction products were separated by SDS–10% PAGE. The gel was stained with Coomassie brilliant blue (lower panel) and then analyzed with a BAS 1500 phosphorimager (upper panel). (F) Binding assay of K369R and wild-type KLF5 with SET. Wild-type and K369R mutant KLF5 fused to GST were immobilized on GST resin followed by a pull-down assay of SET protein. Lanes 1 and 4 are SET input. Lanes 2 and 5 are GST alone. All experiments were done at least twice with consistent findings. +, present; −, absent.

Regulatory pathway through the DBD. Another finding of the present study is that the DBD plays a pivotal role in mediating regulatory interactions. It is well established that the activation and regulatory domain of transcription factors mediates important regulatory interactions (19, 36, 37). In contrast, our findings and those of others suggest that the DBD mediates regulatory interactions particularly for the Sp/KLF family of zinc finger transcription factors. Interaction of the DBD with acetylases has been shown in the past (45), and others have shown interaction with SWI/SNF (12), deacetylase (8), and cell cycle regulatory factor (13, 20) as well as interaction with other zinc finger transcription factors, including Krüppel-like factors (21).

In the present study, we show that there is regulatory interplay by interacting proteins, namely through physical interaction and modification (e.g., acetylation) as mediated by acetylase and its inhibitor (e.g., masking protein) on the DBD. It is tempting to envision that the DBD of at least this subgroup of zinc finger transcription factors mediates a convergence of multiple regulatory pathways contributing to temporospatial regulation of DNA-involved processes ranging from naked DNA to chromatin remodeling to thus affect gene expression. Given that zinc finger transcription factors greatly evolved in genomic complexity in eukaryotes (46), it is likely that they play an important role in specific transcription associated with biological diversification. A better understanding of the actions and regulation of these factors and pathways will add to our understanding of eukaryotic transcriptional regulation.

Functional implications of KLF5-SET interaction. Our results show a biological setting in which the described mechanisms may contribute to transcriptional regulation of gene expression. The temporospatial biological activity of KLF5 may be dictated by the coordinated regulation of its induced expression and the reduced expression of its negative regulator SET. KLF5 was induced and activated by mitogenic stimulation. In contrast, SET was repressed in response to mitogenic stimulation, and importantly, the repression of SET coincided with the induction of KLF5, which is coupled with expression of a downstream gene. In pathological states, SET and KLF5 were both highly expressed, as examined in an experimental model of atherosclerosis. Expression of SET and KLF5 were both increased in neointimal hyperplasia cells, which are proliferative cells induced in response to a pathological stimulus. Given that their coexpression was seen in late stages of pathogenic evolution, we envision that increased SET expression acted to limit the actions of KLF5 so that uncontrolled inappropriate cell proliferation (e.g., oncogenesis) did not occur.

Interestingly, p300 is also induced by phorbol ester stimulation (23). Together with our findings, our proposed mechanism of coupled positive regulation of KLF5 by p300 and negative regulation by SET may play a role in the biological setting of pathophysiological induction of KLF5 as exemplified by the actions of the model agonist, phorbol ester. Induction of KLF5 activation and of its downstream genes are coupled with up-regulation of its coactivator p300 and down-regulation of its repressor SET, thereby likely resulting in amplification of KLF5 actions. Another interesting issue on the regulation of SET may be that there is possible regulation of SET by its splicing variant and dimerization partner TAF-Iα. We have seen that while SET alone is expressed in KLF5-active smooth muscle cells, TAF-Iα is also expressed in stoichiometric amounts in other cells. Given that TAF-Iα has been suggested to be a negative regulator of SET (28), we believe that the amount of SET relative to TAF-Iα may be critical in regulating SET actions. SET may therefore act only in the absence of TAF-Iα or when SET is found in relative abundance; therefore allowing for dissection of the unique actions of SET as a negative regulator in the TAF-Iα-deficient KLF5-expressing smooth muscle cell. Collectively, our findings suggest a new transcriptional regulatory pathway of a DNA-binding transcription factor by combined use of inducible positive and negative cofactors centered on interaction and acetylation which likely reflects the mechanisms underlying triggered activation by stimuli.

Importantly, the regulatory pathway by p300 and SET allows for bimodal regulation of KLF5, which will efficiently allow for a rapid amplification of coordination and fine tuning of gene regulation such as in gene- and cell type-specific transcriptional regulation (e.g., temporal as well as spatial regulation). Future studies aimed at understanding the regulation and generality of the described mechanisms will further advance our understanding of the biological role of the involved factors and reactions.

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S. Miyamoto, T. Suzuki, S. Muto, and K. Aizawa contributed equally to this work.

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Differentiation induction of human promyelocytic leukemia cells by acyclic retinoid (polyprenoic acid)

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Abstract

Acyclic retinoid (all-*trans*-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid) has a slightly different structure from all-*trans* retinoic acid (ATRA), while it binds cellular retinoic acid-binding protein with a similar binding affinity to that of ATRA. We studied the in vitro efficacy of acyclic retinoid for the differentiation induction of human promyelocyte-derived HL-60 cell line and primary cultured blast cells obtained from 8 patients with acute nonlymphocytic leukemia (ANLL) including 3 acute promyelocytic leukemia (APL) patients. HL-60 cells and ANLL cells were incubated with or without retinoids for 5 days. Acyclic retinoid induced the differentiation of HL-60 cells and APL cells at 10^{-6} mol/l, while ATRA induced differentiation at 10^{-7} mol/l. These concentrations were well below those that affected cell growth and viability. Although ATRA has an excellent capacity for differentiation induction of HL-60 and APL cells, it is also known to have severe, sometimes fatal, adverse effects, including retinoic acid syndrome. In contrast, acyclic retinoid is reported to have a much wider safety margin than that of ATRA. A clinical trial of acyclic retinoid for the differentiation induction therapy of APL may be worthwhile.

Key words: Differentiation induction therapy; Acyclic retinoid; APL; HL-60

1. Introduction

Retinoic acid induces terminal granulocytic differentiation of the human promyelocyte-derived cell line, HL-60 [1,2]. Recent studies have confirmed the superiority of all-*trans* retinoic acid (ATRA) over 13-*cis* retinoic acid both in the in vitro induction of neutrophilic differentiation of leukemic cells obtained from patients with acute promyelocytic leukemia (APL) and in clinical efficacy for the treatment of APL patients [3–5]. However, ATRA is also known to have severe, sometimes fatal, adverse effects including 'retinoic acid syndrome' [6]. Hence, more safe retinoids are desired for clinical application.

Acyclic retinoid is a synthetic derivative of

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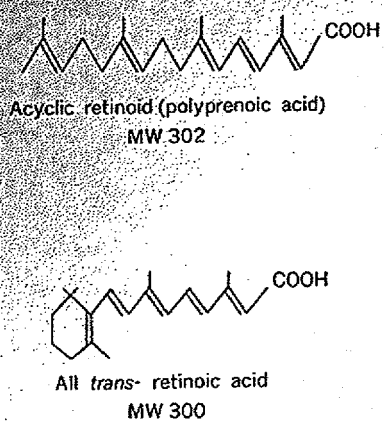

Fig. 1. Chemical structures of acyclic retinoid (E5166) and all-*trans* retinoic acid. MW, molecular weight.

polyprenoic acid, and showed positive results in screening by means of binding assay to cellular retinoic acid-binding protein (CRABP) and cellular retinoid binding protein, type F (CRBP(F)) [7]. Although acyclic retinoid (polyprenoic acid) has a slightly different structure from ATRA (Fig. 1), it binds to CRABP and CRBP(F) with a binding affinity similar to that of ATRA [7]. Acyclic retinoid has been found to inhibit cell growth and to induce differentiation of human hepatoma derived cell line (PLC/PRF/5) [8]. Acyclic retinoid is less toxic than ATRA both in vivo [7] and in vitro [8].

In the present study, we investigated the efficacy of acyclic retinoid for the differentiation induction of HL-60 and leukemic cells obtained from patients with acute non-lymphocytic leukemia (ANLL) including APL.

2. Materials and methods

2.1. Chemicals

Acyclic retinoid (polyprenoic acid or open-chain C_{20} analog of retinoic acid; all-*trans*-3,7,11,15-tetramethyl-2,4,6,10,14-hexadecapentaenoic acid: E5166) was donated by Eisai Co., Tokyo. All-*trans* retinoic acid (ATRA) was purchased from Sigma Chemical Co., St. Louis, MO (Fig. 1). These retinoids were dissolved in absolute ethanol at 10^{-2} mol/l and diluted with Iscove's modified Dulbecco's medium (IMDM; GIBCO, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) to give final concentrations of 10^{-4} to 10^{-9} mol/l for use.

2.2. Cell line

The HL-60 cell line has been maintained in continuous suspension culture in Eagle's minimum essential medium (MEM) supplemented with 10% FCS in a humidified atmosphere containing 5% CO_2 at 37°C. Substitution of the medium by IMDM did not affect the cellular characteristics of cultured HL-60.

2.3. Cells

Fresh ANLL leukemic cells were isolated from the bone marrow blood of 8 ANLL patients after obtaining informed consent. Diagnosis of ANLL was made according to the criteria of the FAB classification [9,10]. The clinical characteristics of the patients are summarized in Table 1. Mononuclear cells were separated by centrifugation on a Ficoll–Hypaque density gradient and were placed in culture immediately. In all isolates, leukemic cells accounted for more than 90% of the collected cells. Chromosome analysis was performed on a minimum of 20 metaphases by a conventional G-banding method, and revealed t(15;17)(q22;q12) in 2 of 3 APL cases. In APL cells, detection of PML/RARα transcripts was performed by RNA-polymerase chain reaction (PCR) [11,12]. The cDNA fragment used for the detection extended from nucleotide 1117 to 1685 of the published PML/RARα cDNA sequence [12]. In all APL cases, PML/RARα transcripts were positive.

2.4. Cell culture of HL-60

A total of 5×10^4 HL-60 cells/ml were plated in 24 multiwell-plates (Becton-Dickinson, Costar, MA). Cells were incubated with or without retinoid for 5 days at 37°C in a humidified atmosphere containing 5% CO_2. After 5 days of incubation, total cell number was determined using a Burker-Tuerk counting plate, and viability by trypan blue dye exclusion test. Cytospin smear slides were then prepared, and subjected to May–Gruenwald–Giemsa staining. The cytogram of at least 200 cells was assessed for each preparation.

Table 1
Clinical characteristics of patients with ANLL

Case	Age (years)	Sex	Diagnosis		Bone marrow		Chromosome analysis		Positive surface markers (CD)
			(FAB)	status	NCC ($\times 10^4/\mu l$)	Bl (%)			
1	46	M	M3	relapse	35.2	45	46XY,t(15;17)	PML/RARα(+)	13, 33
2	49	M	M3	onset	80.0	95	46XY	PML/RARα(+)	13, 33
3	48	F	M3	relapse	13.9	94	46XX,t(15;17)	PML/RARα(+)	N.D.
4	17	F	M2	onset	11.8	91	46XX		13, 33, HLA-DR
5	34	M	M2	relapse	8.9	73	46XY,2p,13q		13, 33
6	41	F	M4	onset	19.9	43	46XX,t(16;21)		13, 33, HLA-DR
7	27	M	M4	onset	18.5	83	46XY,inv(16)		13, 14, 33
8	32	M	M5a	onset	15.5	90	46XY		13, 14, HLA-DR

Bl, blast cell; N.D., not done.

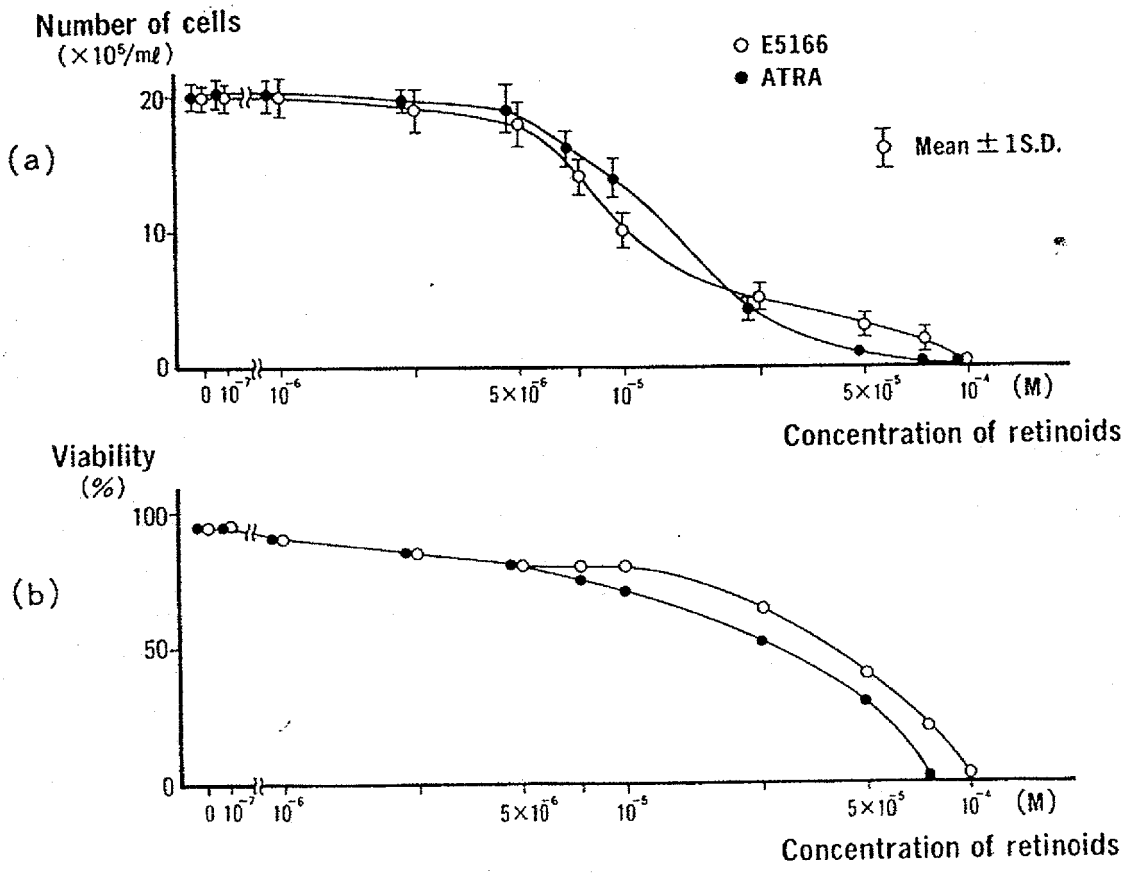

Fig. 2. (a) Inhibitory effects of retinoids on the growth of HL-60 cells. The cells were treated with retinoid for 5 days. (b) Viability of HL-60 cells after exposure to retinoids for 5 days. Viability was determined by trypan blue dye exclusion test. ○, acyclic retinoid (E5166); ●, all-*trans* retinoic acid (ATRA). Values are expressed as mean ± S.D.

Table 2
50% Growth-inhibitory concentration (IC_{50}) and 50% cytotoxic dose (LD_{50}) of retinoids on HL-60 cells

	IC_{50} (mol/l	LD_{50} (mol/l)
E5166	1.0×10^{-5}	3.8×10^{-5}
ATRA	1.4×10^{-5}	2.5×10^{-5}

E5166, acyclic retinoid; ATRA, all-*trans* retinoic acid.

2.5. Culture of leukemic cells of ANLL patients

Culture and analyses of leukemic cells isolated from ANLL patients were performed in a similar manner as described above except the initial cell density was 1×10^6 cells/ml.

3. Results

3.1. Effects of retinoids on cell growth and viability of HL-60

Fig. 2a shows the total cell number of HL-60 after 5 days of incubation with various concentrations of acyclic retinoid and ATRA. Both retinoids reduced the growth rate at concentrations above 7.5×10^{-6} mol/l and completely inhibited growth at 7.5×10^{-5} mol/l of ATRA or 1×10^{-4} mol/l of acyclic retinoid. The IC_{50} values (50% inhibition of cell growth) were 1×10^{-5} mol/l for acyclic retinoid and 1.4×10^{-5} mol/l for ATRA (Table 2). The concentrations that killed 50% of the cells (LD_{50}) were 3.8 × 10- mol/l for acyclic retinoid and 2.5×10^{-5} mol/l for ATRA (Fig. 2b, Table 2).

3.2. Morphological changes of HL-60 induced by retinoids (Table 3)

In the absence of retinoids (control), cultured HL-60 cells were predominantly promyelocytes, with 7–8% mature cells (myelocytes, metamyelocytes, and banded and segmented neutrophils). Striking increases in the population of mature cells, which are characteristic of terminal differentiation of HL-60, appeared at 10^{-6} mol/l for acyclic retinoid and at 10^{-7} mol/l for ATRA ($P < 0.001$ compared with control).

3.3. Effects of retinoids on growth and differentiation of ANLL cells

The effects of retinoids on the growth and differentiation of ANLL cells were examined at a concentration of 10^{-6} mol/l. The limited yield of leukemic cells obtained from clinical ANLL cases forced us to select only a single experimental concentration of retinoids, which was chosen according to the previous data, described above.

Table 3
Cytograms of HL-60 Cells after incubation for 5 days with retinoids

Retinoid	Concentration (mol/l)	Cytogram (%)						Mature cells (%)
		Bl	Pro	My	Meta	Band	Seg	
E5166	1×10^{-8}	2	90	7	1	0	0	8
E5166	1×10^{-7}	1	64	30	5	0	0	35
E5166	1×10^{-6}	1	12	41	19	19	8	87*
ATRA	1×10^{-8}	2	69	26	3	0	0	29
ATRA	1×10^{-7}	1	16	47	20	12	4	83*
ATRA	1×10^{-6}	0	8	42	20	17	13	92*
Control		2	91	7	0	0	0	7

E5166, acyclic retinoid; ATRA, all-*trans* retinoic acid; Bl, blast; Pro, promyelocyte; My, myelocyte; Meta, metamyelocyte; Band, banded neutrophil; Seg, segmented neutrophil.
Mature cells (%) = My (%) + Meta (%) + Band (%) + Seg (%).
*$P < 0.001$ compared with control.

Table 4
Cell number after 5-day culture of ANLL cells with or without retinoids

Case	FAB classification	Cell number ($\times 10^6$ cells/ml)		
		Control	ATRA	E5166
1	M3	1.12 ± 0.08	0.92 ± 0.11	0.95 ± 0.07
2	M3	0.72 ± 0.04	0.78 ± 0.11	0.77 ± 0.10
3	M3	1.20	1.20	1.30
4	M2	0.90 ± 0.06	0.82 ± 0.12	0.85 ± 0.06
5	M2	1.00 ± 0.03	0.92 ± 0.05	0.92 ± 0.06
6	M4	0.92 ± 0.08	0.94 ± 0.08	0.93 ± 0.04
7	M4	2.01 ± 0.09	2.00 ± 0.08	1.98 ± 0.11
8	M5a	0.79 ± 0.05	0.71 ± 0.14	0.75 ± 0.10

The results are shown as mean ± S.D. of triplicate determinations, except Patient 3.
ATRA, all-*trans* retinoic acid; E5166, acyclic retinoid.

Table 5
Cytograms after 5-day culture of ANLL cells with or without retinoids

Case	FAB classification	Treatment	Cytogram (%)						Mature cells (%)
			Bl	Pro/Mono	My	Meta	Band	Seg	
1	M3	Control	4	90	6	0	0	0	6
		E5166	0	12	38	35	12	3	88**
		ATRA	1	9	40	28	17	5	90**
2	M3	Control	3	92	5	0	0	0	5
		E5166	2	14	48	32	2	2	84**
		ATRA	2	14	45	30	8	1	84**
3	M3	Control	5	88	4	1	2	0	7
		E5166	4	17	59	17	3	0	79**
		ATRA	5	15	54	20	4	2	80**
4	M2	Control	92	1	4	0	3	0	7
		E5166	90	2	5	3	0	0	8
		ATRA	94	0	5	1	0	0	6
5	M2	Control	93	0	7	0	0	0	7
		E5166	90	2	5	1	0	2	8
		ATRA	95	1	3	1	0	0	4
6	M4	Control	82	8	5	2	3	0	10
		E5166	80	14	4	1	1	0	6
		ATRA	80	11	2	2	2	3	9
7	M4	Control	82	1	6	2	0	0	8
		E5166	48	25	22	2	2	1	27*
		ATRA	49	20	23	4	3	1	31*
8	M5a	Control	75	12	3	3	4	3	13
		E5166	70	18	2	4	3	3	12
		ATRA	68	15	4	5	4	4	17

E5166, acyclic retinoid; ATRA, all-*trans* retinoic acid; Bl, blast; Pro, promyelocyte; My, myelocyte; Meta, metamyelocyte; Band, banded neutrophil; Seg, segmented neutrophil.
Mature cells (%) = My (%) + Meta (%) + Band (%) + Seg (%).
*$P < 0.01$ compared with control.

Significant reduction in the total number of viable cells was not observed after 5-day incubation with such low concentrations of retinoids (Table 4). In contrast, acyclic retinoid and ATRA induced the majority of leukemic cells to differentiate to mature myeloid cells in all 3 cases with APL (Patients 1–3, Table 5). The increases in the proportion of mature cells induced by retinoids in the 3 patients were statistically significant compared with the control ($P < 0.001$). In other cases with ANLL (Patients 4–8), significant differentiation was not induced by retinoids except in Patient 7, with the diagnosis of myelomonocytic leukemia.

In Patient 1, the effects of retinoids at 10^{-7} and 10^{-8} mol/l on the differentiation of APL cells were also examined. At 10^{-7} mol/l, ATRA induced significant differentiation, while acyclic retinoid did not. At 10^{-8} mol/l, ATRA and acyclic retinoid did not induce differentiation.

4. Discussion

Our results show that acyclic retinoid, as well as ATRA, induces differentiation of both HL-60 cells and leukemic cells obtained from clinical APL cases. Both retinoids induced differentiation at concentrations much lower than those that affected cell growth and viability. Acyclic retinoid required a concentration of 10^{-6} mol/l to induce differentiation with the same efficacy as that of 10^{-7} mol/l ATRA.

In general, the dose of ATRA (45 mg/m^2) commonly used in patients with APL is reported to be well tolerated [13]. However, even this dose is occasionally poorly tolerated in some patients with APL [14,15]. Acyclic retinoid is reported to have a safety margin much wider than that of ATRA [7,8], and it is possible to administer 600 mg/man (approximately 340 mg/m^2) acyclic retinoid daily (unpublished observation of Phase I clinical trial for the chemo-prevention of hepatoma in patients with liver cirrhosis). Phase I plasma pharmacokinetic studies of acyclic retinoid, administered at an oral dose of 600 mg/man, revealed a mean area under the curve (AUC) of 10.05 μg/ml/h. This AUC is approximately 10 times that of ATRA (0.68 μg/ml/h) given at a dose of 45 mg/m^2 [16]. In view of these combined findings, a clinical trial of acyclic retinoid for the differentiation induction therapy of APL may be worthwhile. Regarding the severe adverse effect(s) of ATRA, retinoic acid syndrome [6], it is not yet clear whether or not acyclic retinoid also causes retinoic acid syndrome, and this should be determined in clinical trials.

As to the molecular mechanisms of action of acyclic retinoid, the compound binds both nuclear retinoic acid receptor β (RARβ) and retinoid X receptor α (RXRα) [17]. However, it has not been elucidated whether RARβ and/or RXRα mediates the effect of acyclic retinoid. It is also not clear whether or not acyclic retinoid affects CRABP expression, whose up-regulation is reported to induce the resistance of APL cells to ATRA after long-term administration of ATRA [16]. We are currently conducting further investigations to answer such questions.

5. Acknowledgements

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